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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/147984> since 2016-07-13T09:27:48Z

Published version:

DOI:10.1111/1541-4337.12072

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This is the accepted version of the following article:

Ceuppens, S., Li, D., Uyttendaele, M., Renault, P., Ross, P., Ranst, M. V., Cocolin, L. and Donaghy, J. (2014), Molecular Methods in Food Safety Microbiology: Interpretation and Implications of Nucleic Acid Detection. *Comprehensive Reviews in Food Science and Food Safety*, 13: 551–577. doi: 10.1111/1541-4337.12072

which has been published in final form at [http://dx.doi.org/ 10.1111/1541-4337.12072](http://dx.doi.org/10.1111/1541-4337.12072)

Molecular Methods in Food Safety Microbiology: Interpretation and Implications of Nucleic Acid Detection

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Keywords

Culturing, molecular methods, nucleic acids, DNA, RNA, stability, interpretation, viable bacteria, infectious viruses, ingestion.

Abstract

Because of increasing demand for rapid results, molecular techniques are now applied for the detection of microorganisms in foodstuffs. However, interpretation problems can arise for the results generated by molecular methods in relation to the associated public health risk. Discrepancies between results obtained by molecular and conventional culture methods stem from the difference in target, namely nucleic acids instead of actively growing microorganisms. Nucleic acids constitute 5 to 15% of the dry weight of all living cells and are relatively stable, even after cell death, so they may be present in a food matrix after the food-borne microorganisms have been inactivated. Therefore, interpretation of the public health significance of positive results generated by nucleic acid detection methods warrants some additional consideration. This review discusses the stability of nucleic acids in general and highlights the persistence of microbial nucleic acids after diverse food processing techniques based on data from the scientific literature. Considerable amounts of DNA and RNA (intact or fragmented) persist after inactivation of bacteria and viruses by most of the commonly applied treatments in the food industry. An overview of the existing adaptations for molecular assays to cope with these problems is provided, including large fragment amplification, flotation, (enzymatic) pre-treatment, and various binding assays. Finally, the negligible risks of ingesting free microbial nucleic acids are discussed and this review ends with the future perspectives of molecular methods such as next generation sequencing (NGS) in diagnostic and source attribution food microbiology.

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1. Introduction

Contamination by microbial food-poisoning agents may occur at various stages in the food chain. Contaminants can be present in raw products (animal or vegetable) prior to harvesting or they may gain access during slaughter or processing, by the addition of contaminated food ingredients or processing aids, from the factory environment or by cross-contamination from other contaminated foods or from food handlers (Figure 1). The implementation of the hazard analysis and critical control point (HACCP) approach is pivotal to food safety management. The system includes defining control measure(s) to reduce the foodborne microbial hazard to an acceptable level. In many situations, heat-inactivation may be defined as a critical control point (CCP) to ensure that foodborne pathogens are killed or substantially reduced.

Over the past 25 years, there have been considerable advances in the development and use of molecular techniques for the detection of microorganisms in foodstuffs as a result of the increasing demand for rapid results. These are normally based on detecting specific DNA or RNA target sequences using amplification processes, in particular the polymerase chain reaction (PCR) (Cocolin and others 2011). Their adoption, in many instances, has replaced or supplemented traditional culture detection methods with culture methods still recognized as the gold standard for most bacterial foodborne pathogens. But in the case of some foodborne viruses, which are not culturable, nucleic acid-based assays remain the only choice for their detection. Microbial (bacterial or viral)-derived nucleic acids may enter the food chain from similar reservoirs as indicated for the pathogens themselves. Intact living cells will possess intact DNA/RNA, although dead cells may also house intact nucleic acids. Furthermore, the presence of fragmented extracellular nucleic acids emanating from microbial or viral sources cannot be excluded in foods. For example, adventitious viral nucleic acids have been detected in the porcine-derived trypsin enzyme (Victoria and others 2010). Because nucleic acids are relatively stable and may be present in a food matrix, even after processing steps routinely used in food preparation that can kill viable food pathogens, one must be careful in interpreting the public health significance of their presence in processed foods (Cenciarini-Borde and others 2009; Stals and others 2011). This review aims to

provide evidence of the persistence of microbial nucleic acids through food processes and how they act as the target molecule(s) of molecular methods for pathogen detection. It addresses the interpretation and implications of positive molecular assay findings from a diagnostic and public health perspective. The main aspects of the review are outlined in Figure 1.

2. Use of molecular (nucleic acid-based) methods in food safety microbiology

The gold standards in food microbiology have been conventional culture-based methods. However, there are some constraints which have driven microbiologists to look for alternative methods such as nucleic acid-based detection methods (BOX 1).

(1) The use of molecular techniques are the only resort for the investigation of microorganisms which are impossible or very difficult to culture *in vitro*. For example, it is currently still impossible to cultivate the major food-borne viral pathogen human norovirus and it was only after the development of molecular detection methods that a reliable clinical diagnosis of norovirus infection could be achieved (Payne and others 2012).

(2) Culture-independent methods are independent of the physiological state of the target microorganisms, so stressed, injured, and viable but nonculturable (VBNC) cells can also be detected (BOX 2). For example, conventional methods for *Campylobacter* spp. in stool and food samples require robust bacterial growth on selective agar plates, making these methods unreliable for the detection of stressed cells (Omar and Battie 2012).

(3) The time required for analysis is generally much shorter than that for conventional culture based methods. This is of utmost importance when time is a crucial factor or when the target microorganisms are particularly slow-growing. For example, cultivation techniques for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are not standardized and results are only obtained after 4 to 16 weeks due to the slow growth of this microorganism (Slana and others 2008; Douarre and others 2010).

(4) Molecular techniques offer a solution for atypical strains or strains for which a selective medium is unavailable or not performing well, such as lecithinase- and hemolysin-negative *Bacillus cereus* strains (Fricker and others 2008) and sorbitol-fermenting *E. coli* O157 (Posse and others 2008).

(5) DNA-based detection methods can be extremely specific and detect or differentiate at the level of species, for example, specific detection of *Cronobacter sakazakii* (formerly known as *Enterobacter sakazakii*) in infant formula without detection of closely related *Enterobacter* spp. (Soler and others 2012). Also, highly strain-specific PCR tests exist for the differentiation of probiotic lactobacilli strains (Sisto and others 2009).

(6) Nucleic acid-based detection methods can target specific serotypes, genotypes, or pathotypes based on identified virulence genes or other marker genes. For example, detection of genes encoding shiga toxins and other virulence factors to differentiate generic commensal *Escherichia coli* from pathogenic ones, such as human pathogenic verocytotoxin-producing *E. coli* (VTEC) (Figure 2). Detection of VTEC by culture-based methods is a very complex isolation procedure involving many specialized media (Posse and others 2008; Beutin and others 2009), after which the presence of toxin and virulence genes in the isolates need to be confirmed using other techniques, such as (q)PCR or latex agglutination. Moreover, screening of food and animal feed for the top five most important VTEC serotypes is recommended by qPCR (ISO/TS 13136 2012). Classical O- and H-serotyping of *Escherichia coli* can also be performed by PCR (Wang and others 2003; DebRoy and others 2011).

(7) The presence of other (dominant) populations which may mask the presence of the target organism can be overcome by nucleic acid-based assays. For example, detection of low numbers of human pathogenic *E. coli* (VTEC) in the presence of 10,000-fold more generic *E. coli* in fecal and food samples is possible with real-time PCR (qPCR) (Beutin and others 2009).

Due to the shortcomings of conventional culture-based methods, molecular techniques have become increasingly important in food microbiology over the last few decades, and the numbers of applications and assays continue to rise, mainly because of the increased speed of analysis in comparison with the conventional methods (Postollec and others 2011). A considerable commercial diagnostics market has developed for the detection and quantification of specific microorganisms by targeting sequences of their DNA and/or RNA. Alternative methods, including nucleic acid-based detection approaches, must be validated to ensure that their performance is equal or better in comparison with the conventional reference methods, and this should be carried out following the

specifications of international agreements and standards (FOOD-PCR 2003; ISO 22119 2011a; ISO 22118 2011b). Specifically for food microbiology, the main applications are qPCR detection, identification, characterisation, or quantification of food-borne pathogens, but also of food spoilage or beneficial bacteria (fermentation cultures and probiotics). The current trend is to combine detection or quantification of several different pathogens in one (food) sample in the same reaction tube using multiplex (q)PCR. For example, multiplex qPCR for the simultaneous detection of human norovirus of genotypes GI and GII was developed (Stals and others 2009). Moreover, reverse transcriptase (real-time) PCR (RT-(q)PCR) applications are now being developed to monitor the growth and metabolic activity of food bacteria, which can be used to control industrial processes such as fermentation, and also spoilage, and to perform risk analysis in the case of studying the responses and virulence of pathogens in food during processing and storage. For example, the gene expression response of food-borne pathogens *Salmonella* and VTEC to heat and salt stress was monitored in meat (Kjeldgaard and others 2011). RT-qPCR has also revealed that the expression levels of virulence genes *inlA* and *inlB* were higher in clinical *Listeria monocytogenes* strains than in nonclinical strains and was accompanied by higher *in vitro* invasion of Caco-2 and HepG2 cells (Werbrouck and others 2006, 2007).

However, nucleic acid detection techniques suffer from specific drawbacks: these have been elsewhere reviewed in more in detail (Cocolin and others 2011; Postollec and others 2011; Uyttendaele and others 2014). Moreover, microbiological specifications have historically been defined by bacterial numbers based on culture methodology. In brief, the shortcomings of nucleic acid-based methods are:

- (1) The costs are generally higher than those for conventional microbiological methods, including laboratory infrastructure requirements in addition to the reagents and various equipment requirements.
- (2) Inhibition of the PCR reaction by food components is a frequently encountered problem, although it can be monitored by internal amplification controls and circumvented by optimization of the sample preparation.
- (3) The limit of quantification is often rather high, in the range of 10 to 1000 bacteria/viruses per g or mL. Thus for bacterial pathogens in food, presence/absence testing after enrichment is often more appropriate than direct quantification. For pathogens which cannot be cultured (such as human noroviruses), methods focus on concentration of the target pathogen from the sample into a smaller

volume prior to their detection or quantification. In these cases, it is essential that the food matrix components, which may have inhibiting or interfering effects, are removed by proper sample preparation.

(4) Depending on the application, it may be a disadvantage that no isolates are obtained with molecular methods. Especially in the clinical and epidemiological setting, a bacterial isolate is required for further characterization (for example, determining antibiotic susceptibility), typing, and source tracking (as in outbreak investigations), as well as further research.

(5) The most important drawback is probably the detection of naked DNA and DNA from dead microorganisms, which can lead to difficulties in interpretation, especially in a food quality control situation. This is most problematic in the study of nonculturable (viral) pathogens such as human noroviruses, since their infectious status cannot be evaluated by solely nucleic acid detection (Li and others 2011; Knight and others 2013). Several solutions exist in the form of sample pretreatments and modified PCR protocols to assist in the interpretation of results (Table 1, BOX 1, BOX 3).

3. Stability of nucleic acids

Nucleic acids are spontaneously degraded in solution by hydrolysis. RNA is particularly susceptible to such degradation because its phosphodiester bond is less stable than that of DNA (Lindahl 1993). However, the *N*-glycosyl bonds of DNA are less stable than those of RNA, so DNA is more susceptible to depurination (loss of the bases adenine and guanine) and to a lesser extent to depyrimidination (loss of the bases cytosine and thymine). Moreover, DNA can be damaged by hydrolytic deamination, oxidation, and alkylation, thus leading to aberrant base derivatives, mutations, lesions, and ultimately decomposition of DNA. DNA and RNA can also be degraded by extracellular (microbial) DNases and RNases, which are released from dead microorganisms and secreted by live ones to gain nutrients (Palchevskiy and Finkel 2006, 2009). Several studies have indicated that the metabolic activity of microorganisms is the major factor for free nucleic acid degradation in the environment (Romanowski and others 1992; Tsai and others 1995; Deere and others 1996; Wetz and others 2004).

Free DNA is more stable at low temperatures, under dry conditions (including (partial) dehydration), in the absence of oxygen, in the absence of living microorganisms, and when the DNA is absorbed to particles (Lindahl 1993; Lorenz and Wackernagel 1994). More specifically, the persistence of DNA from inactivated microorganisms in food depends not only on their initial concentration and the degree of protection offered by the dead structure (and thus the type of inactivation) but also on the temperature, the food matrix, and the presence of other microorganisms during storage after inactivation (Dupray and others 1997; Herman 1997). Similarly, the degradation of RNA in microorganisms after lethal treatments depends on the type of transcript, that is its specific stability and its relative abundance, the type and the severity of the inactivation treatment, the type of microorganism, the physiological condition of the population prior to inactivation, and the subsequent holding conditions (Sheridan and others 1998; Sung and others 2005; Cenciarini and others 2008). Ribosomal RNAs are among the most persistent transcripts, but the decay of different mRNAs after bacterial death is not generally predictable.

4. Effect of food processing on nucleic acid stability

Many food processing operations have an impact on the stability of microbial DNA and RNA and thus influence the detection of nucleic acids remaining after the treatment. The bacterial membrane potential and/or its structural integrity is one of the primary targets for inactivation and preservation treatments applied during food processing (Dock and Floros 2000). The secondary effect of such damage tends to be the loss of macromolecules, including nucleic acids, from the cell interior. In addition to the passive release from dead or injured microorganisms, nucleic acids are also actively secreted from living bacteria (Lorenz and Wackernagel 1994). As a result, substantial extracellular levels are present in foods, but these are susceptible to spontaneous and enzymatic degradation (Section 3). Additionally, processing may lead to inactivation without disintegration of the bacterial or viral structure, whereby nucleic acids remain within the inactivated microorganism.

During the processing of foods, many processes are intended to substantially reduce or eliminate bacterial or viral pathogens. Verification of validated processing steps is generally performed by culturing. Frequently, molecular methods, in combination with (pre)enrichment, are also used.

However, without (pre)enrichment, nucleic acid-based methods may give positive signals despite the inactivation process. The persistence of naked and bacteria- and virus-bound nucleic acids after various lethal treatments is covered in Tables 2, 3, and 4. Only studies which assessed the presence of nucleic acids simultaneous with the determination of bacterial viability or the viral infectivity were included in the compilation of these Tables. In addition, only results of complete inactivation studies were considered in order to allow conclusions about the degradation of nucleic acids after bacterial death or viral inactivation, which is not possible when residual numbers of surviving bacteria or infectious viruses are still present. Since *in vitro* culturing of human noroviruses is currently not possible and the only marker for human norovirus persistence and survival is RT-qPCR data (Knight and others 2013), information on viral inactivation (and infectivity) studies versus stability of nucleic acids is restricted to cultivable surrogate viruses.

Important factors influencing DNA and RNA persistence include: the initial concentration of microorganisms or nucleic acids, the type of inactivation treatment, and the matrix (either food, food suspensions or buffers), as detailed in the Tables. The size of the amplified DNA or RNA sequence is crucial for the interpretation of PCR-positive results, since long sequences disappear relatively quickly after microbial inactivation. In contrast, small fragments of microbial nucleic acids will remain present for a longer time throughout the degradation process of the genomic material. For example, inactivation of *Streptococcus gordonii* by penicillin could be monitored by RT-qPCR of a 427 bp fragment of the 16S rRNA gene, but not by a smaller 119 bp sub-fragment of this region, because only the presence of the large fragment was correlated with intact RNAs and bacterial viability (Aellen and others 2006). Since DNA damage is associated with reduced recovery and amplification of large fragments, simultaneous quantification of multiple fragments of different sizes can be performed to estimate the extent of DNA damage (Murray and others 2007). Especially for nonculturable viruses, it is an interesting strategy to amplify large or even genomic size fragments to detect only undamaged virus genomes (Rodriguez and others 2009; Knight and others 2013). In addition, the specific target sequence for amplification is also important, because the stability of nucleic acids is dependent on the sequence, namely, the stability is increased for sequences with a high G(C) content due to stronger

hydrogen bonds and the formation of stable quadruplex structures (Chen and others 2007; Joachimi and others 2009). Sequence stability can be exploited in specific applications, such as detection of the most unstable genomic region of the hepatitis A virus and the poliovirus, namely the 5' NTR, correlated well with that of infectious viruses after inactivation with chlorine dioxide (Li and others 2002; Simonet and Gantzer 2006). Under certain conditions, the food matrix may exert protective effects against degradation, for example, during boiling of soymilk the DNA remained stable (Kharazmi and others 2003a). Conversely, the food matrix is a source of nucleases and may also decrease the stability of nucleic acids, such as the stability of *C. jejuni* 16S rRNA gene was decreased on chicken skin (Uyttendaele and others 1997).

4.1. Thermal treatments

Heat treatments are among the most commonly used food processing operations, either alone or in combination with other processes. High temperatures cause degradation (depurination or deamination) and fragmentation of the (free) nucleic acid molecules, but not necessarily complete degradation (Lindahl 1993; Gryson 2010), even after extreme processing such as baking and autoclaving (Table 2). Dry heat causes less DNA damage (fragmentation) than steam treatment at the same temperature (Chiter and others 2000). Nicking and fragmentation of DNA is strongly increased under acid conditions (pH 4.0), because of the strongly increased depurination rate at low pH (Lindahl 1993; Bauer and others 2003). Generally, DNA degradation is thus expected to be most pronounced in acidic heat-treated food products.

DNA degradation kinetics in heat-inactivated bacteria showed an exponential decrease of DNA amount (Nogva and others 2000). Frequently applied heat treatments in the food industry, such as pasteurization, yield dead microbial cells with relatively intact genomic DNA and even RNA, which can still be detected by (RT)-(q)PCR (Tables 3 and 4). Ribosomal RNA is not easily destroyed, because the secondary structure of rRNA and the associated ribosomal proteins protect it from degradation by endogenous or exogenous nucleases (McKillip and others 1998). Moreover, if high initial numbers of bacteria (6 to 7 log CFU/mL) were present, even the most unstable nucleic acids,

such as mRNAs, are rarely completely degraded by pasteurization (65 °C for 30 min) (Cenciarini and others 2008).

In general, naked viruses are more stable to heat treatment than enveloped viruses (Carter and Saunders 2007). Since most of the food-borne viruses are small and non-enveloped particles (Koopmans and Duizer 2004), only naked viruses are discussed here. Heat-inactivation of viruses is reported as a continuous process during which damage to both capsid proteins and viral nucleic acids is inflicted, resulting in loss of infectivity, capsid dissociation, and RNA exposure (Knight and others 2013). Heat-inactivation of viruses shows significant strain variation, but it occurs faster at high temperatures (≥ 50 °C) (Bertrand and others 2012). The temperature-sensitivity is lower in complex matrices than in simple matrices, suggesting a protective effect of molecules such as proteins, fats, and carbohydrates. However, loss of infectivity of heat-inactivated viruses is not correlated with nucleic acid degradation, possibly because ribonucleoprotein particles (RNPs) are formed by re-association of capsid proteins and RNA following heat treatment (Baert and others 2008; Li and others 2011; Bertrand and others 2012). It has been concluded that heating primarily damages viruses by capsid denaturation without complete destruction of the capsid and the nucleic acids (Pecson and others 2009), leading to false positive results obtained by RT-qPCR (Table 4). For example, pasteurization (heating at 70 °C for 2 to 3 min) effectively inactivated murine norovirus 1 (MNV-1), but the genomic copy number estimations of murine and human noroviruses by RT-qPCR were unaffected (Baert and others 2008; Li and others 2011). On the other hand, for low(er) concentrations, (RT)-(q)PCR detection of nucleic acids may correlate well with the presence of infectious viruses, especially for the most unstable viruses, such as inactivation of 4 log plaque-forming unit (PFU)/mL feline calicivirus (FCV) by chlorine (Table 4).

4.2. Nonthermal treatments

Many forms of mechanical processing, including grinding, milling, blending, homogenization, mixing, punching, and extruding, are often applied during food processing. Depending on the type of mechanical processing, shear forces cause small to extensive damage of the nucleic acids both in the food matrix itself and in the food microbiota (Gryson 2010). Furthermore, grinding or chopping of

food results in the release of nucleic acids and nucleases from the food and from the microorganisms present. Subsequently, naked nucleic acids, from whatever source, become susceptible to degradation by the released nucleases (Klein and others 1998).

Aside from thermal and mechanical processing, irradiation, high pressure, sonication, disinfectants, and lytic agents such as phages, are also applied during food processing to achieve substantial reduction of pathogens. Obviously, cleaning and sanitizing agents are applied to eliminate microbial contaminants. These nonthermal processes often cause inactivation of microorganisms and leave behind relatively large fragments of nucleic acids, detectable by (RT)-(q)PCR (Tables 3 and 4). For example, large 16S rRNA gene fragments (> 1400 bp) were still present after complete inactivation of 6 log CFU/mL *E. coli* O157:H7 and *Staphylococcus aureus* by UV irradiation (McKillip and others 1998). Similarly, the viral capsid appears to be the primary target for UV, leaving genomic RNA fragments still available for RT-qPCR detection (Nuanualsuwan and Cliver 2002). As illustrated in Tables 3 and 4, considerably more information is available for thermal inactivation of pathogens and its consequences for nucleic acid persistence in comparison with nonthermal processes.

Virulent bacteriophages can be applied for food decontamination, to control susceptible pathogenic bacteria in foods or to control bacterial spoilage, because they can be very effective in the targeted elimination of specific pathogens under specific conditions (EFSA 2009). It should be noted that in this type of inactivation treatment, bacteriophages need to be applied in relatively high doses, > 7 log PFU/cm² to eliminate *Listeria monocytogenes* on a cheese surface during ripening (Carlton and others 2005). The moisture content of a food influences the mobility of the bacteriophage, and the physiological and nutritional status of the host bacterium determine the efficacy of bacteriophage action. Successful application of bacteriophages leads to high levels of phages and thus viral DNA in the food. It may also cause leakage of bacterial DNA into the food matrix. Although the microbial host DNA has been reported to be rapidly degraded and incorporated into progeny phages during the infection (Powell and others 1992; Wikner and others 1993), the extent of the breakdown or fragmentation has not been characterized. Moreover, care should be taken in regard to transduction (transfer of bacterial DNA from cell to cell inside viral capsids) and the transfer of viral genes into the host genome via lysogenic phages.

5. Interpretation of molecular analytical methods

The notable stability of DNA in the environment is illustrated by the recovery of DNA and successful amplification by PCR from archaeological and paleontological samples which can be thousands of years old (Landweber 1999). Although this technology is very useful for the detection, identification and quantification of genetically modified (GM) ingredients in processed foods, in the field of food safety microbiology, the detection of DNA must be carefully interpreted. Amplification by PCR of DNA originating from pathogenic microorganism(s) does not infer the presence of live populations of the pathogen in the tested food and thus does not of itself constitute a food safety risk.

The prerequisite for application of any method is its accurate design and thorough control, both beforehand and during the routine application, to safe-guard the quality of the obtained results. Controls on appropriate performance and implementation of PCR are necessary to exclude interpretation problems due to the technical nature and complexity of the PCR technique. Appropriate interpretation of results requires that the validation of the sensitivity and the specificity of the (q)PCR method are studied before its application and that controls to check the nucleic acid amplification process are routinely applied during each analysis (ISO 22174 2005; ISO 20837 2006; ISO 20838 2006; ISO 22118 2011; ISO 22119 2011). In particular, for food and environmental samples, appropriate controls to monitor the efficacy of the critical steps in the sample preparation and nucleic acid amplification are required to exclude false negative and positive interpretations of results (D'Agostino and others 2011). Quantitative qPCR assays require good calibration curves, constructed by extraction of standard quantities of the target microorganisms in the relevant physiological state from the relevant (food) matrix (Ceuppens and others 2010).

5.1 Variances between culture and molecular methods

In the absence of technical problems yielding false positive and false negative results, all remaining interpretation problems of molecular results originate from the fact that conventional and molecular methods do not detect the same targets, namely, actively growing microorganisms and their nucleic acids, respectively. Obviously, negative results are obtained by both culture and molecular methods

when neither target microorganisms nor their nucleic acids are present in the sample (Figure 3). Similarly, unequivocal positive results arise where infectious viruses and viable bacteria are detected by culturing as well as by PCR based methods. However, difficulties in interpretation of results can arise when contradictory results are obtained by molecular and culture methods.

Negative results can be obtained by molecular methods when the target microorganism(s) are present in forms or in microenvironments which pose difficulties for nucleic acid extraction. For example, bacteria present in biofilms, in the form of spores or internalized into plant materials, may impair nucleic acid extraction and result in negative results (or severely underestimated numbers of genomic copies), despite the inherent sensitivity of the qPCR reaction itself (Lear and others 2010; Ceuppens and others 2010; DiCaprio and others 2012). Moreover, the cell wall structure of some microorganisms can present difficulties for recovery of DNA and thus requires optimization of the DNA extraction, for example, *Mycobacterium avium paratuberculosis* (Donaghy and others 2008). Most of these issues can be overcome by optimization of the sample preparation, but can pose problems when standard extraction protocols – often optimized with widely used Gram-negative or Gram-positive bacteria as pure cultures of vegetative cells in suspensions or artificially inoculated foods – are applied without validation of their performance for a specific application. Many molecular (PCR-based) methods require a (pre)enrichment step (up to 24 h) to enhance levels of target microorganism(s) to detectable levels. To minimize negative results when detecting low numbers of potentially injured bacteria, it is important that the enrichment step is optimised in terms of medium composition and incubation time (Jasson and others 2009). Many food processing techniques result in injury, if not death, of bacterial or viral cells. Moreover, the food matrix itself is often a sub-optimal environment for microorganisms, containing stressed and injured cells. A sublethally injured bacterium is damaged by a stress, but given appropriate conditions can recover by repairing the damaged components. Consequently, injured cells often display a prolonged lag phase in growth/recovery under nonselective culture conditions and may not be able to grow at all under a selective environment. They may have additional or more stringent requirements for repair and growth than those encountered in standard enrichment broths. As a result, the enrichment procedure may fail to reach the required minimal numbers for detection in the case of sublethally injured cells. For

example, low levels of sublethally injured *Salmonella enterica* in chocolate could not be recovered after enrichment in buffered peptone water (BPW), unless BPW was supplemented with milk powder, independent of the detection method, such as plating, PCR or enzyme-linked fluorescent assay (ELFA) (Jasson and others 2011).

One of the most common issues encountered in food pathogen diagnostics is the detection of positive PCR results in parallel with negative culture results or, for example, if concurrent *in vitro* culture confirmation is technically not possible with the molecular detection method. The detection of microbial nucleic acids poses difficulties in terms of interpretation, since the presence of genomic fragments is not necessarily correlated with viable bacteria and infectious viruses (Tables 3 and 4). For example, RT-qPCR is most commonly used to detect human noroviruses, because these viruses cannot be cultured *in vitro*, but no discrimination can be made with standard RT-qPCR between infectious viruses, defective viruses, and free RNA (Knight and others 2013). Inconsistent results can arise for a number of reasons. Molecular methods are able to detect nongrowing target microorganisms, namely nonculturable microorganisms (Amann and others 1995) and stressed, sublethally injured, and VBNC forms of culturable bacteria (BOX 2). For example, fecal coliforms have been shown to enter into and emerge from the VBNC state during wastewater treatment, which presents a potentially dangerous underestimation of public health risks when culture-based tests are used to assess suitability of biosolids, such as as soil amendments (Taskin and others 2011). Furthermore, some pathogens are difficult to isolate by culturing regardless of their physiological status, for example, isolation rates are usually low for VTEC (Joris and others 2011). Additionally, nucleic acid-positive results are obtained by the detection of free nucleic acids and those from inactivated bacteria and noninfectious viruses. Naked DNA from inactivated microorganisms may be present in food, water, the processing environment, and even as an adventitious contaminant in enzymes or processing aids (for example, if derived as products from industrial bacterial fermentations). Free nucleic acids are degraded in the environment and in food matrices, but short fragments can persist for considerable times outside their microbial host cell (Table 2). Moreover, nucleic acids are additionally protected inside inactivated microorganisms, which increases their persistence even further (Tables 3 and 4). For example, bacterial infections can be detected and identified in a fast and sensitive way by PCR amplification of

bacterial 16S rDNA followed by sequencing, but residual DNA of inactivated pathogens may still be detected in the patient's body long after successful treatment and recovery. For example, *Streptococcus pneumoniae* DNA was detected during replacement of a bioprosthetic heart valve in a patient who had suffered from pneumococcal endocarditis 7 years earlier without any evidence of recurrent infection (Branger and others 2003). Viral nucleic acids are also frequently detected after viral particles have lost infectivity, such as DNA from noninfectious adenoviruses that was detected in approximately 16% of water samples from 2 southern California urban rivers (Choi and Jiang 2005). Naked viral nucleic acids can be transmitted by cell lines, animal sera, and tissues and then contaminate the derived products, for example, porcine pancreas-derived trypsin (Victoria and others 2010). Moreover, the application of virulent bacteriophages as processing aids, to reduce surface contamination of food with specific pathogens or to prevent bacterial food spoilage, introduces large quantities of viral DNA into the food product (EFSA 2009).

Detection of a specific genotype characterized by multiple virulence genes or other genetic markers pose interpretation problems if the target genes also occur separately in the microbial population. Since nucleic acids from all microorganisms are pooled during sample preparation and extraction, it cannot be ascertained whether the detected genes originated from one microorganism of the targeted genotype or from multiple strains which contain one of those genetic signature sequences. For example, detection of VTEC causing severe disease in humans targets VTEC which possess at least one verocytotoxin gene (*stx1* and/or *stx2*) in combination with the intimin adhesion gene (*eae*) and belonging to the top 5 of clinical serogroups O157, O26, O111, O103 and O145 (EFSA 2013); although exceptions exist, such as the O104:H4 strain responsible for the outbreak in Germany in 2011. PCR-positive results for both verocytotoxin and intimin genes in the sample suggest the presence of pathogenic VTEC, but it is also possible that a mixture of nonpathogenic *E. coli* strains (one with only verocytotoxin genes and another with only the adhesion gene) has generated the positive signals. Culture confirmation of PCR-positive results to demonstrate the presence of all genes in one isolate are hampered by the fact that VTEC strains easily lose their toxin genes during sub-cultivation and long-term storage (Joris and others 2011) and also in the human body during infection (Mellmann and others 2005), since the *stx* genes are encoded on prophages in the *E. coli* chromosome.

Detection of genomic presence of virulence genes only indicates the potential for pathogenicity, because it is the expression level of those genes, possibly in combination with others, that make a strain hazardous. For example, the majority of *Bacillus cereus* strains harbor at least one toxin gene in its genome, while not all strains are pathogenic (Ceuppens and others 2011). However, conventional culturing methods offer no possibility to distinguish between pathogenic and benign strains of a species, while molecular methods do, that is, detecting gene expression (mRNA) with RT-(q)PCR rather than genes (DNA) with (q)PCR.

5.2 Strategies to facilitate interpretation of nucleic acid-based methods

The detection of a target microorganism by a nucleic acid-based assay may be confirmed by culturing for most bacteria. However, it is not possible for all microorganisms, particularly nonculturable viruses. This difficulty in interpretation is exemplified by the high prevalence of human norovirus which has been obtained by RT-qPCR in different matrices: 3.9 to 59.1% in shellfish (Woods and Burkhardt 2010; Lowther and others 2010), 4.2 to 45% in water (Lodder and others 2010; Borchardt and others 2012; Allmann and others 2013), 23.3% in cherry tomatoes (Stals and others 2011), 6.6 to 40.0% in soft red fruits (Baert and others 2011; Stals and others 2011), and 28.2 to 50.0% in leafy greens (Baert and others 2011). Improper interpretation of such data can lead to misconceptions of public health risk. Fortunately, various adaptations to the conventional (RT)-(q)PCR protocols have been developed to avoid or alleviate the detection of free nucleic acids, noninfectious viruses, and dead bacteria (Table 1). Since free nucleic acids and those in inactivated microorganisms are prone to fragmentation, detection of intact DNA or RNA from viable bacteria or infectious viruses can be attempted by amplification of larger fragments and/or multiple fragments from different genomic regions, although the latter results in lower amplification efficiencies and thus lower sensitivity (Baert and others 2008; Schnetzinger and others 2013). In order to avoid the detection of viral DNA/RNA fragments, the whole genome could be amplified and/or positive results could be confirmed by transfection assays (Baert and others 2008).

Viable/dead stains such as ethidium or propidium monoazide (EMA or PMA) can be applied prior to PCR to exclude free nucleic acids and those present in damaged bacterial cells or viral capsids from

PCR detection, since cell membrane integrity is essential for bacterial viability and an intact virus capsid for virus infectivity (Figure 4, BOX 1, BOX 3). EMA- and PMA-PCR have been widely and successfully applied to detect or quantify viable microorganisms, including bacteria in the vegetative and spore form, yeasts, and fungi (Vesper and others 2008; Rawsthorne and others 2009; Rawsthorne and Phister 2009; Taskin and others 2011; Shi and others 2012; Zhu and others 2012; Elizaquivel and others 2012; Radulovic and others 2012; Crespo-Sempere and others 2013; Gensberger and others 2013; Singh and others 2013; Dinu and Bach 2013; Blooi and others 2013; Schnetzinger and others 2013). However, EMA and PMA can also diffuse into living bacterial cells with intact membranes under certain conditions, albeit at lower efficiency (Nocker and others 2006). Some studies have suggested that in some cases dead bacteria may possess intact cell membranes, since not all inactivation methods target the cell membrane (Joux and Lebaron 2000; Jofre and Blanch 2010). Moreover, PMA-PCR could no longer be applied to determine living *Staphylococcus aureus* cells in swab samples resulting from the increased PMA diffusion in viable cells due to the drying and wiping processes (Schmidlin and others 2010). Since EMA and PMA require double-stranded DNA/RNA or single strands with extensive secondary structures, EMA/PMA pre-treatment has been applied with variable success to detect infectious virus particles (Fittipaldi and others 2010; Parshionikar and others 2010). PMA-RT-qPCR was successfully applied to differentiate between infectious and noninfectious coxsackievirus, poliovirus, and echovirus after inactivation with hypochlorite and heat (37 °C and 72 °C), but not after inactivation at 19 °C (Parshionikar and others 2010). Similarly, PMA-qPCR was able to distinguish infectious bacteriophage T4 particles from noninfectious ones after heat-inactivation at 110 °C but not at 85 °C (Fittipaldi and others 2010). Therefore, PMA pre-treatment is suitable for detecting infectious viruses after defined inactivation treatments and can contribute as a research tool studying the efficacy of virus inactivation, but monitoring of infectious viruses in the environment and subsequent public health implications requires caution because of the variable performance of this method.

Enzymatic pre-treatment (with DNases and/or RNases) has been applied to degrade free nucleic acids, as well as those present in damaged viruses, thus excluding them from subsequent molecular detection (Figure 5). To overcome the RNase resistance of RNA present in ribonucleoproteins (RNPs),

simultaneous treatment with RNase and proteinase K has been performed, but the combined use of these enzymes is difficult to control since proteinase K can also degrade RNase and intact capsids. Moreover, application of enzymatic treatment with proteinase K and RNase prior to nucleic acid extraction and detection decreased but did not eliminate the false positive detection of noninfectious viruses (Baert and others 2008; Pecson and others 2009).

Specific binding of the viruses by receptors or other binding ligands prior to (RT)-(q)PCR has been performed to exclude free nucleic acids and damaged virus particles from detection. For example, binding to Caco-2 cells, pig gastric mucin, and BSA prior to RT-qPCR reduced the signal 100- to 1,000-fold for human noroviruses (Li and others 2011). To discriminate clinically significant viral contamination, definition of critical acceptance limits for the number of viral genomic copies detected in food products was also proposed based on epidemiological and contamination data (Woods and Burkhardt 2010; Dore and others 2010; Neuhaus and others 2013; Stals and others 2013). Immuno-capture (RT)-(q)PCR entails specific binding of bacteria or viruses by antibody-bound magnetic beads, (immuno-magnetic separation or IMS), prior to PCR, which is useful to eliminate free nucleic acids from PCR detection. However, antibody-based binding cannot distinguish between infectious and noninfectious viruses and such methods would not exclude capture of dead cells, where the immune separation target is membrane-based. An exception to this constraint has been reported by Solve and others (2000). Peptide-mediated magnetic separation (PMS) can also be applied prior to PCR detection. Detection of ≥ 2.7 log CFU/mL *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bulk milk samples by PMS-PCR was achieved by PMS using a conserved surface protein followed by PCR detection with MAP specific primers (Stratmann and others 2006). Performing IMS or PMS prior to PCR as a sample preparation step is useful to eliminate the detection of free nucleic acids, although in most cases intact target cells are captured.

Specific qualitative detection of viable bacteria can be achieved by implementing an enrichment or pre-enrichment step prior to molecular detection where, given the sample dilution factor and the detection limit of the PCR-based method, a positive signal is indicative of the presence of viable organisms. Moreover, specific detection of mRNA or rRNA can be attempted to target viable bacteria, but these molecules remain available for detection after bacterial death for some time (Table 3, BOX

1). Flotation prior to qPCR has also been applied to distinguish viable bacteria from free DNA and dead bacteria, for example, for viable *Campylobacter* in chicken rinse samples (Wolffs and others 2005a). Until now, flotation has not been applied to viruses, although discrimination of intact virus particles of large species with multiple capsid layers seems theoretically possible. Exclusive detection of viable bacteria can also be achieved by bacteriophage amplification assays (BOX 1) (Favrin and others 2001; Foddai and others 2010). Although many of the methods mentioned above have shown great potential to diminish positive results arising from free nucleic acids, noninfectious virus particles, and/or dead bacteria, strict differentiations have not generally been achieved, especially in food matrices. Case-specific solutions should be tailored to the application's context to ensure optimal performance of molecular methods and correct interpretation of the generated positive results.

6. Implications of microbial DNA ingestion

Nucleic acids make up a considerable proportion of dry cell weight. Humans ingest large quantities of animal and vegetable DNA and RNA daily through food. Animal tissues contain 10 to 20 g DNA per kg dry weight, plant tissues 0.6 to 3 g DNA/kg, and bacteria approximately 30 g/kg (Rizzi and others 2012; Delgado and others 2013). Through the consumption of fermented foods considerable amounts of microbial nucleic acids are consumed on a daily basis. Most foods consumed by humans are not sterile but are compliant with microbiological criteria, with respect to foodborne pathogens and/or process hygiene criteria. Food manufactured through process steps, validated to eliminate or substantially reduce the microbial/pathogen load, may contain nucleic acids derived from pathogenic microbial/viral sources, be detectable by nucleic acid-based methods, and be ingested as part of the food matrix. A possible consequence of ingestion of nucleic acid, as a risk factor, is linked to their transfer into a microbial cellular environment that allows expression of information encoded in the nucleic acids.

During ingestion, DNA from most cells present in food is degraded/cleaved to nucleotides or small fragments and taken up as metabolites by intestinal cells in the small intestine, while the majority of the remaining fraction (0 to 3.7%) is completely degraded in the large intestine (Netherwood and others 2004; Mazza and others 2005). For example, extensive digestion of RNA (89%) and DNA

(80%) occurred in the bovine small intestine and was accompanied by the transient appearance of nucleic acid building blocks (Mc Allan 1980). However, a fraction of the ingested DNA can avoid complete degradation in the gastrointestinal tract, including free plasmid DNA (Wilcks and others 2004). Despite the majority of studies showing degradation of foreign DNA in the gastrointestinal tract, foreign DNA uptake in the gastrointestinal tract may be a natural process, similar to the absorption of metabolites (Palka-Santini and others 2003). Studies have reported the presence of microbial and dietary plant DNA in the blood and tissues of animals (Beever and Phipps 2001). For example, microbial DNA from the gastrointestinal lumen was taken up in the white blood cells of mice, the uptake of soybean DNA into the white blood cells and milk of cows, and chloroplast DNA transmitted to muscle, liver, spleen, and kidney of chickens (Schubbert and others 1994; Klotz and Einspainer 2000). Recently, large DNA fragments (> 10,000 bp) of edible plants were also shown to persist in human blood (Spisak and others 2013).

Although free DNA may be temporarily available in the digestive tract, naked DNA is unable to express its function in the intestinal tract unless it has been taken up and integrated into a host or bacterial cell by transformation. Despite application of the worst-case conditions, no successful natural transformation events of bacteria or host cells in the gastrointestinal tract with recombinant DNA from genetically modified (GM) feed have been observed in all *in vivo* studies performed so far, although the possibility of such events has been demonstrated under optimized conditions *in vitro* and in food (Jonas and others 2001; Chambers and others 2002; Kharazmi and others 2003b; Rizzi and others 2012). For example, *Bacillus subtilis* acquired a plasmid conferring erythromycin resistance by transformation in chocolate milk (Zenz and others 1998). In contrast, the probability of transformation in the gastrointestinal tract is strongly reduced, possibly due to degradation of the donor DNA and/or failure of the recipient strain to develop competence *in vivo* (Kharazmi and others 2003b). No direct evidence for natural transformation events of bacteria with non-GM DNA in the gastrointestinal tract has been demonstrated, except for the transformation of *S. pneumonia* cells (nonpathogenic R forms to pathogenic S forms by heat-killed S donors) in the intestines of mice (Griffith 1928). Although there is evidence that many bacterial species develop specific states allowing for DNA acquisition, there are factors limiting the extent of such acquisition for transfer and expression in the environment (Lorenz

and Wackernagel 1994; Brigulla and Wackernagel 2010). First, the quality of DNA may not be sufficient to ensure its functionality for transformation, as suggested by the lack of detectable DNA uptake by isolated gut bacteria grown in vitro, and by *Acinetobacter baylyi* in the rat model (Nordgård and others 2007). Second, transformation is dependent on particular environmental conditions. This limitation is believed to be the reason for the failure of transfer of recombinant DNA to *Streptococcus gordonii* in a model system in foods and in gnotobiotic rats (Kharazmi and others 2003). Another investigation also led to the conclusion that gut microbiota were not prone to acquire markers added in the diet, even when selective pressure was added. This restriction was influenced by DNA homologies between donor DNA and potential docking sites in recipient DNA, thus allowing integration in the genome and expression (Nordgard and others 2012). In conclusion, the likelihood of gene transfer in the gut from naked DNA to intestinal microbiota is very small, so it is assumed that the risks for transferring antibiotic resistance genes, virulence genes, genetically modified genes, or other functional coding nucleic acid fragments to intestinal bacteria are negligible in case of free DNA present in food products. It must be stressed that this conclusion is drawn only for transformation with free DNA in the gastrointestinal tract. Horizontal gene transfer between living bacteria, for example, conjugation, and mediated by phages, for example, transduction, is considerably more probable and may occur in the gastrointestinal tract (Marchesi, 2011). Conjugation presents the highest risk for the horizontal distribution of genes, in particular those encoding antibiotic resistance and virulence, since these genes are often present on mobile elements such as plasmids and transposons (Mathur and Singh 2005; Verraes and others 2013). Furthermore, the probability of antimicrobial resistance transfer by means of conjugation may be higher for stressed donor and recipient bacteria under food preservation stress conditions (Mc Mahon and others 2007). Finally, bacteriophages are environmental reservoirs for the horizontal transfer of antimicrobial resistance genes and may spread such genes among susceptible bacteria (Nakaminami and others 2007; Muniesa and others 2013).

7. Metagenomics: from microbial ecology to food microbial diagnostics?

Currently, PCR is the method of choice for detection of nucleic acids in food, although, due to its high specificity, this technique can only detect well-defined targets, the specificities of which can be

predicted on the basis of the sequences that are used for primers and reporter probes. The advances in high-throughput sequencing (HTS) methodologies and their concomitant use in metagenomic studies and culture-independent diagnostics offer considerable potential (Ercolini 2013). These HTS technologies include the 454 Roche pyrosequencer (producing about a million sequences of 400–500 base length), Illumina, or solid sequencing technologies (producing over a billion sequences of 50–100 base length), and other platforms such as Ion Torrent and PacBio (Thomas and others 2012). The outputs could be a mixture of bacterial 16S rRNA genes, of which tens to hundreds of thousands of copies are sequenced, or random DNA fragments corresponding to microbial DNA, or retro-transcripts of RNA molecules of which millions of short sequences are determined. The sequences generated can be analyzed using bioinformatics to permit taxonomic assignment of the bacterial content of the original sample. The power of these methods is that they can easily sequence the entire DNA content of an ecosystem, for example a food sample, and are already extensively applied in microbial ecology and gene expression analysis studies (BOX 4).

From the generated sequences, based on 16S rRNA gene amplification from the metagenome, information on the microbial taxonomy of culturable and nonculturable organisms in the test matrix/sample can be obtained. While currently applied primarily for the study of the microbiota composition from an ecology perspective, theoretically such information could provide information on the presence of undesirable microorganisms in a food sample. However, for use in a diagnostic setting, standardization and adoption of best practices would be required in terms of sample preparation, optimized qualitative and quantitative nucleic acid extraction, sequence data handling, storage, and sharing. Furthermore, similar interpretation issues will arise, as described for traditional nucleic acid amplification tests, for example, differentiation of viable and nonviable microorganisms in the food sample (Josephson and others 1993). Also, depending on the nature of the food (raw or processed), the level of target pathogen(s) will be low or absent relative to other microbiota, therefore sequence coverage and resolution could limit detection of minor genera or species. Increasing the number of sequences will increase the cost per sample and increase the volume of data for bioinformatics analysis (Segerman and others 2011) – both being considerable hurdles for routine application of HTS in the diagnostic analysis of food. From a food safety microbiology perspective, these sequencing

strategies are likely to remain more expensive than PCR against a small number of targets, and will therefore more likely provide molecular data needed to facilitate source tracking and outbreak detection, and improve discovery of emerging subtypes, for example, the public database Food Microbe Tracker (Vangay and others 2013), particularly where implicated isolates are available.

8. Conclusion

Introduction of culture-independent nucleic acid-based methodologies in food microbiology has solved existing problems associated with conventional culturing techniques and has led to interesting new applications. However, interpretation of the results from nucleic acid detection in the context of food safety is not always straightforward, since nucleic acids have been shown to persist after inactivation of microorganisms. Food may contain free microbial nucleic acids of detectable fragment size and inactivated microorganisms with intact nucleic acids, which are detected by molecular methods. Several strategies and modified molecular techniques have been developed to cope with the differential detection of viable and nonviable bacteria and infectious and noninfectious viruses. Although much progress has been made, no perfect solution is currently available for each situation. Nevertheless, molecular techniques, including the more recent advances in high-throughput sequencing and metagenomics have opened up a new world of applications and possibilities and will undoubtedly continue to contribute to the future development of food diagnostic and ecology technology and scientific knowledge.

Several aspects which are important for the interpretation of microbial nucleic acid detection are not currently fully understood or studied in depth. The effect of new or alternative processes to enhance food shelf-life and improve safety such as high-pressure treatments, enzymatic treatments, pH alterations, irradiation, and so on, on the integrity of microbial cell structure and nucleic acids after inactivation of microorganisms remains to be elucidated. The influence of food matrices, that is, their intrinsic properties and the role of food-derived nucleases, on the stability and fragmentation of (free) nucleic acids during food processing and post-processing also needs further investigation. The influence of stress factors encountered in the food chain on the induction of the VBNC state in bacteria and their subsequent stress resistance and virulence should be determined to further

characterize the health threat posed by pathogens in the VBNC state. More knowledge on the occurrence of RNPs, a type of noninfectious virus particles, in the environment, and in food is required to aid interpretation of positive (RT)-(q)PCR results.

BOX 1: Molecular methods and modifications

Conventional Polymerase Chain Reaction (PCR) is a culture-independent alternative technique to detect microorganisms by *in vitro* amplification of a fragment of its DNA and visualization of these DNA end products on agarose gels using ethidium bromide (Mullis and others 1986). As an alternative for conventional PCR, loop-mediated isothermal amplification (LAMP) was developed (Notomi and others 2000). The advantages are increased specificity due to the use of 4 different primers binding to 6 different regions of the target DNA sequence, decreased reaction time (< 1 h) and lower costs because no thermal cyclers are required for this isothermal reaction. Quantitative real-time PCR (qPCR) is an improvement of the conventional PCR, during which detection and amplification of DNA production occurs simultaneously by adding nonspecific fluorescent dyes such as SybrGreen to the mastermix or by fluorescent dyes coupled to specific DNA sequences (for example probes) (Higuchi and others 1993). As a result, qPCR is faster and more sensitive than conventional PCR and has the potential to yield quantitative results when combined with appropriate calibration curves. qPCR is more efficient when small fragments (100 to 300 bp) are amplified, but this also makes the technique more susceptible to detect free DNA fragments instead of only DNA extracted from an intact microorganism. DNA may still be present after bacterial death, such as in bacteria which were inactivated by boiling, autoclaving, drying, disinfectants, and starvation (Table 3). When it is important to detect only viable bacteria, or when the target microorganisms are RNA viruses, RNA becomes the target molecule instead of DNA (Figure BOX 1). The most suitable target for exclusive detection of viable bacteria is messenger RNA (mRNA), because it is only present in living cells and it has a very low half-life of several seconds to minutes (Sheridan and others 1998; Rauhut and Klug 1999; Keer and Birch 2003). However, mRNA fragments persist for several hours after heat-inactivation of bacteria, so they can still be detected by RT-(q)PCR for some time after bacterial death (Table 3). mRNA detection is more complex than that of DNA, because it is more unstable, it is

difficult to extract in sufficient quantities from (small numbers of) bacteria in food matrices and contamination with its corresponding homologous DNA must be avoided. Ribosomal RNA (rRNA) can be detected instead of mRNA to detect cells which display general metabolic activity, since it reflects the rate of protein synthesis, is present in much larger quantities than mRNA and is much more stable (Deutscher 2006). However, due to its higher stability, rRNA is not suitable to indicate bacterial viability after mild inactivation processes. rRNA accurately reflected viability after severe heat treatments such as autoclaving, but it persisted in dead cells after moderate heat treatment and UV irradiation (McKillip and others 1998). For RNA amplification, Reverse Transcriptase (real-time) PCR (RT-(q)PCR) can be performed by starting with an additional step during which the RNA is converted into complementary DNA (cDNA) using the enzyme Reverse Transcriptase (RT) (Verma and others 1973). Next, the amplification and detection of this cDNA may occur with conventional PCR, but most frequently by real-time PCR (qPCR), since this assay is more sensitive and allows quantification of the initial RNA, which is often required, such as for assessing microbial gene expression levels. Alternatively, RNA can be detected by Nucleic Acid Sequence-Based Amplification (NASBA) at a constant temperature by 3 enzymes, namely RT, RNaseH, and T7 RNA polymerase (Compton 1991). An alternative method to only amplify DNA from viable cells consists of application of intercalating stains such as ethidium monoazide (EMA) and propidium monoazide (PMA) prior to (q)PCR (Nogva and others 2003; Rudi and others 2005). Since EMA and PMA do not penetrate viable cells with intact membranes, only the amplification of free double-stranded DNA and DNA from damaged and dead cells is prevented by irreversible binding of photo-activated EMA/PMA to DNA and subsequent precipitation of the EMA/PMA-DNA complex. Performing magnetic separation of the target microorganism prior to PCR as a sample preparation step can eliminate detection of free nucleic acids, but nonviable bacteria and noninfectious viruses may still be detected, since antibodies also bind to inactivated microorganisms. Magnetic separation of the target microorganism from others and from inhibitory sample components not only isolates but also concentrates the target organisms, thereby improving selectivity and sensitivity simultaneously. Binding of the target microorganisms to paramagnetic beads is achieved by coating the beads with polyclonal antibodies towards the target microorganism, that is, immuno-magnetic separation (IMS), or with ligands to specific proteins

present on the target microorganism, that is, peptide-mediated magnetic separation (PMS). Flotation prior to qPCR has also been applied to distinguish viable bacteria from free DNA and dead bacteria, for example, for viable *Campylobacter* in chicken rinse samples (Wolffs and others 2005a). Flotation is a one-step centrifugation with a discontinuous buoyant density gradient method, based on the known buoyant densities of living cells, to exclude dead cells and free DNA together with as much of the sample components and the background microbiota as possible. Exclusive detection of viable bacteria can also be achieved by bacteriophage amplification assays. Specific lytic bacteriophages infect only viable target bacteria, while unbound phages are removed by subsequent washing and/or disinfection steps. The phages replicate in the target bacteria, after which the progeny phages are recovered and detected or even enumerated by suitable assays using susceptible host cells, such as plaque assays, changes in optical density, and so on. For example, the phage detection system FASTPlaqueTBTM assay (Biotec Laboratories Limited, UK) has been commercialized for the detection of viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Specificity and performance can be enhanced by combining bacteriophage amplification assays with prior IMS or PMS (Foddai and others 2010). Another type of sample preparation to avoid detection of free DNA and RNA is performing a prior enrichment step. However, this is only feasible in the framework of rapid detection, meaning applicable for detection (not for quantification) of microorganisms which are easily cultured (and not for slow-growing, injured, VBNC, or noncompetitive microorganisms, since these will be overgrown). In conclusion, different strategies exist to assess bacterial viability by molecular methods, which are differently affected by the mode of bacterial inactivation and the prevailing environmental conditions thereafter. Therefore, no single method is universally appropriate, and simultaneous determination of multiple parameters by different methods provides the most reliable detection and/or quantification of viable bacteria.

BOX 2: Viable but nonculturable (VBNC): Bacteria between life and death

Bacteria can be present in various physiological states, ranging from viable to dead over some intermediate stressed states including (sublethal) injury and VBNC (Wesche and others 2009). Adverse conditions such as spray-drying, acidification, oxidation, irradiation, salting, heating, cooling,

and freezing, are frequently encountered stresses by bacteria during food processing. When subjected to minor stress, bacteria can adapt completely to the changed conditions and resume growth at the normal rate (Wesche and others 2009). Adaptation to sublethal stress (stress response) often induces resistance to subsequent exposure to other types of stress and/or higher levels of the same stress. Some pathogenic bacteria are also known to express virulence factors and attain higher virulence after stress exposure. For example, *Salmonella* Typhimurium was more virulent and invasive after heat challenge (Sirsat and others 2011; Nielsen and others 2013) and *Listeria monocytogenes* after acid stress (Neuhaus and others 2013). When more severe but not lethal, then stress causes sublethal injury, and bacteria can only recover if suitable resuscitation conditions are provided. Sublethally injured cells are often defined as cells that are able to grow on nonselective media but not on selective media due to increased sensitivity towards the conditions and/or compounds in the selective medium (Besse and others 2000; Jasson and others 2007). As a result, injured bacteria may present problems for detection and enumeration with conventional culturing due to growth inhibition or retardation on selective media or in enrichment broths prior to detection, leading to false-negative results. Universal recovery conditions probably do not exist, since optimal conditions for resuscitation vary among different species and the stress type encountered (Besse and others 2000; Wesche and others 2009). The VBNC state is the reversible physiological state in which bacterial cells are no longer culturable under their standard conditions but remain alive, that is, they exhibit intact cell membranes and maintain low levels of gene transcription and metabolic activity (Oliver 2010). So in contrast to sublethally injured bacteria, growth is never possible, not even on nonselective media. Transformation to the VBNC state is a survival strategy induced by stressful adverse environmental conditions such as starvation, temperatures outside the normal growth range, decreased and elevated osmotic and oxygen concentrations, hydrogen peroxide, heavy metals, and light. For example, low water temperatures (< 13 °C) induce the VBNC state in *Vibrio parahaemolyticus* (Nowakowska and Oliver 2013) and starvation induces a similar response in *Campylobacter jejuni* (Klancnik and others 2009). The VBNC state is a well-known survival and dissemination mechanism for water-borne bacteria (Deere and others 1996). Concerns exist for food-borne bacteria as well, since many food preservation techniques have been found to induce the VBNC state, for example, potassium sorbate in *Listeria monocytogenes*

(Cunningham and others 2009), sodium bisulfite in cider fermenting *Lactobacillus hilgardii* (Quiros and others 2009), and gamma irradiation in *E. coli* O157:H7 (Lacroix and others 2009). Many animal and plant pathogens exhibit the VBNC state, including *Campylobacter* spp., *E. coli* (including EHEC strains), *Francisella tularensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. (Oliver 2010). Interestingly, VBNC cells of several pathogens still showed expression of virulence genes and remained pathogenic, for example, *Campylobacter jejuni* (Moorhead and Griffiths 2011; Chaisowwong and others 2012), enteropathogenic *E. coli* (Pommepuy and others 1996; Liu and others 2010; Lothigius and others 2010), and *Shigella dysenteriae* (Rahman and others 1994), while other pathogens were shown to become avirulent in the VBNC state, for example, *Listeria monocytogenes* (Cappelier and others 2005), *Aeromonas hydrophila* (Maalej and others 2004), *Vibrio parahaemolyticus* (Coutard and others 2005; Coutard and others 2007), and *Salmonella* (Clausen 2001). Despite the controversy of the ability of pathogens in the VBNC state to cause disease, it has been shown for some that after resuscitation they will recover as fully infectious pathogens, for example, *Vibrio cholera*, (Colwell and others 1996), *V. parahaemolyticus* (Baffone and others 2003), *C. jejuni* (Cappelier and others 1999), enteropathogenic *E. coli* (Aurass and others 2011), and *Aeromonas hydrophila* (Maalej and others 2004). Resuscitation from the VBNC state may occur after simple reversal of the inducing stress factor, for example, increased temperature for *Salmonella enterica* (Gupte and others 2003), undefined bacterial–host interactions (Colwell and others 1996; Cappelier and others 1999), extracellular bacterial proteins, resuscitation-promoting factors (Rpfs) in *Mycobacterium smegmatis* (Shleeve and others 2004), and possibly even quorum-sensing molecules, such as enterobacterial autoinducer AI in *Salmonella enterica*, pathogenic *E. coli*, *Citrobacter freundii*, and *Enterobacter agglomerans* (Reissbrodt and others 2002). It has been hypothesized that the VBNC state is the dormant state of nonsporulating bacteria, from which they awake randomly due to stochastical variations in master regulator gene expression (Epstein 2009). Depending on the environmental conditions these awakened “scout” cells die, if adverse conditions still prevail, or they live, if conditions have changed to growth-permissible ones. At periodic random moments they will thus attempt to resume growth and start a new population. This hypothesis was supported for both

vegetative cells and spores in the absence of germination factors (Buerger and others 2012). The resistance of VBNC cells to environmental and food processing stresses is not well known and appears to be variable, ranging from more sensitive to more resistant, depending on the microorganism and the nature of stress applied (Oliver 2010). However, *Vibrio parahaemolyticus* in its VBNC state was more resistant to heat treatment (50 °C for 60 min), ethanol (13% for 60 min), high salinity (5 M NaCl for 120 min), acidic (pH 3 for 25 min) and alkaline challenge (pH 10 for 60 min), oxidative stress (0.2 mM H₂O₂ for 60 min), antibiotics (100 µg/mL ampicillin and 5 µg/mL chloramphenicol for 240 min), and heavy metals (3.4 mM ZnSO₄·7H₂O for 60 min) (Nowakowska and Oliver 2013). Moreover, VBNC pathogens are resistant to antibiotics targeting growing and metabolically active cells and present a means for pathogens to cause recurrent infections after antibiotic treatment (Rivers and Steck 2001; Lleo and others 2003). For food-borne pathogens, more research is thus warranted to investigate the influence of stress factors encountered in the food chain on the induction of the VBNC state, stress resistance, and virulence. In conclusion, the VBNC state represents a source of living bacteria which are not detected by conventional culture methods. The growing demand of consumers to implement milder food processing may increase the presence of bacteria in VBNC states in processed food, including pathogens (Leistner and Gorris 1995). Although it is currently unclear whether pathogens in the VBNC state remain virulent, and thus whether they are a significant health threat, they should be regarded as potentially hazardous until proven otherwise.

BOX 3: Infectivity of viruses and their detection with nucleic acid-based methods

Virus suspensions are always a mix of infectious viruses with defective virus particles and free viral nucleic acids, even those obtained from natural infections (Knight and others 2013). Non-infectious viruses range from intact defective virus particles, partially degraded capsids containing nucleic acids called ribonucleoprotein particles (RNPs) and intact particles with damaged genomes to free capsid proteins and free viral nucleic acids (Figure BOX 3). As a result, considerably higher concentration estimates of virus suspensions are generated by nucleic acid quantification (genomic copies) than by infectivity assays, for example, murine norovirus 1 (MNV-1) suspensions demonstrated at least 100-fold more RNA copies by RT-qPCR than the plaque forming units (PFUs) in the plaque assay (Baert

and others 2008; Li and others 2011). The particle-to-PFU ratio is the total number of virus particles divided by the number of infectious viruses and presents a measure of the infectious particles in a suspension, but it must be noted that the PFU may present an underestimation of the infectious viruses due to aggregation of virus particles. Many factors influence virus aggregation and the relative abundance of the infectious and other particles in the virus suspension, including the host cell, the virus strain, the virus structure, the suspension matrix, the pH, the ionic strength and the time of harvest in the infection cycle (Persson and Gekas 1994; Langlet and others 2007; Teunis and others 2008; Pinto and others 2010). Interestingly, virus stability is increased in complex matrices such as feces and food (Topping and others 2009; Bertrand and others 2012).

The majority of standard virus extraction methods co-extract free viral nucleic acids and subsequently subject these to the (RT)-(q)PCR assays. As a result, both (small) genomic sequences and nearly intact genomes, for example with only one or a few strand breaks, will be detected with RT-qPCR, which typically amplifies only small fragments (50 to 200 bp). Although intact viral genomes are likely to be degraded in the environment, small viral sequences and ribonucleoprotein particles (RNPs) can persist long after the capsid has been destroyed, so their detection yields positive results in the absence of infectious virus particles (Tsai and others 1995; Choi and Jiang 2005; Dancer and others 2010).

Infectious virus particles have an intact genome and an intact capsid, so adaptations to the RT-qPCR protocol can be made to attempt exclusive detection of infectious viruses based on these 2 characteristics (Table 1). As an alternative to (RT)-(q)PCR, viruses may be detected by enzyme-linked immuno-sorbent assays (ELISAs) or dissociation enhanced lanthanide fluorescent immuno-assay (DELFI) (Kavanagh and others 2011), but the challenge with these tests will lie in the use of conserved antigenic epitopes and the improvement of the sensitivity of the assay (current detection limit is 5 log particles per reaction). Furthermore, these assays also do not distinguish between infectious and defective viruses (Hardy and others 1995). Electron microscopy is the only technique which can differentiate intact particles from damaged capsid shells and remnants thereof, but the detection limit is also very high, 6 to 7 log particles.

BOX 4: Next-generation sequencing (NGS) in food microbiology

Technological advances in sequencing have made it possible to profile an entire complex microbial community by high-throughput next-generation sequencing (NGS) rather than detecting a few of its specific members by (q)PCR or the most abundant fractions by denaturing gradient gel electrophoresis (DGGE) ($\geq 1\%$ of the microbial population and about $\geq 3 \log$ CFU/mL). NGS is the first method which allows complete identification of the microbiota of a food sample and robust saturated diversity analysis of a food microbial community in a single sequencing run of 10^6 to 10^9 reads of heterogeneous DNA fragments of 50 to 600 bp (Bokulich and Mills 2012). These reads typically consist of bacterial 16S rRNA gene fragments, which are then taxonomically assigned and analyzed using bioinformatics. Alternatively, retro-transcripts (cDNA) of microbial RNA molecules can be analyzed to specifically characterize the viable and/or active microbiota in the sample. For example, NGS showed that the spoilage-associated microbiota on beefsteaks originates from the carcasses, but is also established as the resident microbiota in the butchery environment from which it further contaminates the meat (De Filippis and others 2013). Whole genome sequencing of new species and additional strains of the same species for which reference genome sequences are already available can be applied to explore genomic diversity within these microbial species and enhance knowledge on microbial phylogeny and genome evolution (Solieri and others 2013). NGS also makes genotype-phenotype association mapping feasible for complex microbial phenotypes, enabling the identification of the genetic basis of complex phenotypes, the engineering of new phenotypes and the combination of beneficial phenotypes in industrial hosts. For example, whole genome sequencing of commercial *Saccharomyces cerevisiae* strains for wine and beer brewing led to the discovery of 20 new putative genes (Borneman and others 2011). NGS also revealed the importance of multiple bacterial species in dental caries besides *Streptococcus mutans* and provided candidate probiotic strains which inhibited the growth of these cariogenic bacteria from people who had never suffered from caries (Belda-Ferre and others 2012). High-throughput community profiling by NGS provides deeper insight into food fermentations at a system level. Such knowledge can be the basis for rational and whole-genome-assisted choice of starter cultures and probiotics (Solieri and others 2013). For example, NGS was applied to identify the sources of the microbiota involved in the fermentation of premium-quality water buffalo mozzarella, showing that a few thermophilic lactic acid bacteria from the natural whey

culture drive the fermentation, while the raw milk microbiota does not develop (Ercolini and others 2012). NGS also identified new taxa and revealed higher diversity in the artisanal cheese microbiota than was previously known (Quigley and others 2012). Moreover, identification of the pathways or enzymes responsible for significant food processes, simultaneous quantification of bacteria and fungi during food fermentation processes and prediction of the growth and survival of desirable and undesirable microorganisms can lead to enhanced management strategies for fermentation control (Solieri and others 2013). Food meta-genomics, that is, sequencing the collective microbial genomes in food samples, may identify the genes responsible for characteristic properties and functionalities such as probiotic activity, flavor formation, and taste development. Food meta-transcriptomics, that is, collective gene expression analysis in food, can clarify microbial behaviors in food ecosystems. Moreover, sequencing of RNA will reveal information on the viable and metabolically active microbiota. Transcriptomics by NGS is more sensitive and quantitative than by micro-array analysis. Furthermore, NGS transcriptomics does not require prior sequence knowledge, so it is able to cover the full dynamic range of microbial gene expression.

Author Contributions

Conception, planning, and interpretation of the paper was done by JD, LC, PRen, Pros, and MVR, followed by organizing, writing and interpretation performed by SC, DL, MU and JD

Acknowledgment

The authors declare that there are no conflicts of interest. We are grateful to the European branch of the International Life Sciences Institute (ILSI Europe) for permission to modify and update a report entitled “Safety implications of the presence of nucleic acids of inactivated microorganisms in foods” for publication in Comprehensive Reviews in Food Science and Food Safety. This report was made by the expert group consisting of JD, LC, PRen, Pros, and MVR, who received funding from the ILSI Europe Emerging Microbiological Issues Task Force. Industry members of this task force are listed on the ILSI Europe website at www.ilsieurope.eu. For further information about ILSI Europe, please email info@ilsieurope.be or call +32 2 771 00 14.

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Table 1: Possible strategies to improve the detection of viable, injured, and VBNC bacteria and infectious viruses by reducing or even eliminating the positive signal generated by free nucleic acids, noninfectious viruses, and dead bacteria.

Strategy	Free nucleic acids	Noninfectious viruses	Dead bacteria
Culture confirmation [#]	✓ Applicable	✓ Applicable	✓ Applicable
Prior enrichment [#]	✓ Applicable	✗ Not applicable	✓ Applicable
Secondary large fragment amplification	✓ Applicable	✓ Applicable	✓ Applicable
Prior EMA/PMA treatment	✓ Applicable	✓ Applicable	✓ Applicable
Prior enzymatic treatment with nucleases (with/without proteinase K)	✓ Applicable	✓ Applicable	✗ Not applicable
Immuno-capture-PCR: prior antibody based binding, including IMS	✓ Applicable	✗ Not applicable	✗ Not applicable
Prior receptor binding assays, including PMS	✓ Applicable	✓ Applicable	✗ Not applicable
(m)RNA detection	✓ Applicable	✗ Not applicable	✓ Applicable
Prior flotation	✓ Applicable	✗ Not applicable	✓ Applicable

[#]If *in vitro* culturing of the microorganism is possible.

Table 2: Persistence of free nucleic acids (DNA and RNA) after application of various food processing techniques.

Treatment type	Treatment conditions	Storage conditions	Nucleic acid detection	Reference
DNA				
Heat	Autoclaving of soybean DNA (15 µg/mL)	None	Three-fold decrease in the abundance of 80 bp target fragments after 20 min of autoclaving, 30-fold decrease after 40 min, 400-fold decrease after 60 min and 80,000-fold decrease after 80 min	(Debode and others 2007)
	Heating of soybean DNA (15 µg/mL) to 99 °C for 1 to 7 h	None	Severe DNA degradation, but no decrease in the abundance and thus detection of 80 bp target fragments (the mean size of the DNA segments was about 400 bp)	(Debode and others 2007)
	Heating of <i>E. coli</i> plasmid DNA pSG100 (480 µg/mL) at 37 °C and 65 °C for 90 min in 10 mM Tris-HCl with pH 4.0 and in tomato serum with pH 4.3	None	Degradation to fragments of < 1754 bp by all treatments	(Bauer and others 2003)
	Heating of <i>E. coli</i> plasmid DNA pSG100 (480 µg/mL) at 85 °C in 10 mM Tris-HCl with pH 8.4 for 90 min	None	DNA fragments of 1754 bp persist	(Bauer and others 2003)
	Heating of genomic DNA of transgenic maize Bt176 (48 µg/mL) at 85 °C at pH 8.4		Degradation to fragments of < 1416 bp after 30 min and 60 min and to fragments of < 1255 bp after 90 min	(Bauer and others 2003)
Microwaves	Exposure of soybean DNA (15 ng/µL) to microwaves of 800 W for 0 to 15 min	None	Severe DNA degradation, but no decrease in the abundance of 80 bp target fragments	(Debode and others 2007)
Sonication	Exposure of soybean DNA (15 ng/µL) to sonication at 170 W	None	Three-fold decrease in the abundance of 80 bp target fragments and 20- to 30-	(Debode and others 2007)

for 5 min to 8 h		fold decrease of 170 bp target fragments		
Storage	Genomic and plasmid DNA of <i>Yersinia enterocolitica</i> (between 1 mg/mL and 1 µg/mL)	Incubation in chicken rinse at 4 °C and 20 °C	Degradation of 90% of the 300 bp fragments after 8 h at 20 °C and after 9.5 h at 4 °C and degradation of 90% of the 600 bp fragments after 0.5 h at 20 °C and after 1.5 h at 4 °C	(Wolffs and others 2005)
		Incubation in chicken homogenate at 4 °C and 20 °C	Degradation of 90% of the 300 bp fragments after 1 h at 20 °C and 4 °C and degradation of 90% of the 600 bp fragments after 0.5 h at 20 °C and after 1.5 h at 4 °C	
		Incubation in pork rinse at 4 °C and 20 °C	Degradation of 90% of the 300 bp fragments after 120.5 h at 20 °C and degradation of 90% of the 600 bp fragments after 26.5 h at 20 °C and after 35 h at 4 °C	
		Incubation in pork homogenate at 4 °C and 20 °C	Degradation of 90% of the 300 bp fragments after 38.5 h at 20 °C and degradation of 90% of the 600 bp fragments after 74.5 h at 20 °C and after 93.5 h at 4 °C	
	Degradation of <i>E. coli</i> pUC18 plasmid DNA (250 µg/mL) and sugar beet chromosomal DNA (250 µg/mL)	Incubation in raw beet juice at 4 °C, 37 °C and 70 °C	DNA was completely degraded at 70 °C within 10 min, while intact DNA persisted for 10 min at 37 °C and for ≥ 20 min at 4 °C	(Klein and others 1998)
	Chromosomal DNA of maize (20 µg/mL)	Incubation in maize silage effluent at ambient temperature	DNA fragments of 1914 bp persist for 5 min and 684 bp fragments for 60 min	(Duggan and others 2000)
		Incubation in ovine saliva at 39 °C	Fragments of 850 bp and 1914 bp persist for 1 h and fragments of 350 bp and 684 bp persist for 24 h	
		Incubation in	Fragments of 1914 bp,	

		ovine rumen fluid at 39 °C	850 bp and 350 bp persist for ≤ 1 min	
	<i>Legionella pneumophila</i> DNA (180 ng/mL)	Incubation in sewage effluent at 16 °C	DNA fragments of 186 bp and 108 bp persist for ≥ 4 days	(Palmer and others 1993)
	<i>Salmonella</i> Typhimurium DNA (14 to 22 ng/mL, corresponding to 5 to 6 log CFU/mL)	Incubation in seawater at 10 and 20 °C	DNA fragments of 284 bp persist for 3 to 8 days at 10 °C and for 2 to 4 days at 20 °C	(Dupray and others 1997)
	<i>Legionella pneumophila</i> DNA (180 ng/mL)	Incubation in ocean water at 16 °C	Degradation of DNA to fragments of < 186 bp after 2 days and < 108 bp after 3 days	(Palmer and others 1993)
	<i>Aeromonas salmonicida</i> DNA (10 ng/mL)	Incubation in fresh water with sandy and loamy sediment at 13 °C	Degradation of DNA to fragments of < 423 bp after 10 days in water, but DNA fragments of 423 bp persist for 13 weeks in the sandy and loamy sediment	(Deere and others 1996)
		Incubation in loamy sand soil at 23 °C	Fragments of 1029 bp persist for 60 days in loamy sand soil at 3.3% of the initial concentration	
		Incubation in clay soil at 23 °C	Fragments of 1029 bp still present after 60 days in clay soil at 11.2% of the initial concentration	
	<i>E. coli</i> plasmid pUC8-ISP DNA (0.2 µg/g)	Incubation in silty clay soil at 23 °C	Degradation of DNA to fragments of < 1029 bp in the silty clay soil after 10 days	(Romanowski and others 1992)
		Incubation in sterilized (ethylene oxide-treated) soils at 23 °C	Fragments of 1029 bp still present after 60 days in sterilized soils at 100% of the initial concentration	
RNA				
Storage	Free poliovirus RNA (30 ng/mL)	Incubation in seawater at 4 °C and 23 °C	Degradation of RNA to fragments of < 394 bp after 3 days at 23 °C and after 14 days at 4 °C	(Tsai and others 1995)

	Incubation in filter- sterilized seawater at 4 °C and 23 °C	Degradation of RNA to fragments of < 394 bp after 35 days at 4 °C and 23 °C
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Table 3: Persistence of bacterial nucleic acids (DNA and RNA) after application of complete inactivation processes resulting in negative culture results.

Treatm ent type	Treatment conditions	Storage conditions	Nucleic acid persistence post treatment	Reference
DNA				
Heat	Inactivation of 7.5 log CFU/mL <i>E. coli</i> cells in nutrient broth by boiling (unspecified time – temperature conditions)	In pond water at 4 °C	Degradation of DNA to fragments of < 179 bp after 3 weeks	(Josephson and others 1993)
	Inactivation of 8 log CFU/mL <i>E. coli</i> O157:H7 in skim milk by heating at 60 °C for 3 h	In skim milk at 23 °C	DNA was partially degraded but fragments of 266 bp persisted after 48 h storage	(McKillip and others 1999)
	Inactivation of 6 log CFU/mL <i>E. coli</i> in phosphate buffer by heating for 30 min at 60 °C and autoclaving (15 min at 121 °C)	In the dark in phosphate buffer at 20 °C	DNA fragments of 147 bp persist	(Villarino and others 2000)
	Inactivation of approximately 5 log CFU/mL <i>Salmonella</i> Typhimurium in tryptic soy broth by boiling (100 °C) for 10 min	In the dark in filtered (3 µm) seawater at 10 °C	DNA fragments of 472 bp persist for 103 days	(Dupray and others 1997)
		In the dark in seawater at 10 °C	DNA fragments of 472 bp persist 10 to 55 days	
		In the dark in seawater at 20 °C	DNA fragments of 472 bp persist 11 days	
	Inactivation of approximately 9 log CFU/mL <i>Listeria monocytogenes</i> in Ringer solution by heating at 60	In Ringer solution at room temperature	DNA fragments of 702 bp persisted for 90 days with 60 °C and 100 °C at 1% of the initial concentration, while DNA was	(Herman 1997)

	°C for 30 min, at 100 °C for 10 min and at 124 °C for 10 min and autoclaving (124 °C for 15 min)		degraded to fragments < 702 bp after 1 h with 124 °C and autoclaving	
	Inactivation of approximately 7 log CFU/mL <i>Campylobacter jejuni</i> in water by heating at 55 °C, 72 °C and 100 °C for 5 min, 1 h, 6 h, 24 h and 5 days	None	DNA was degraded to fragments < 86 bp after boiling for 24 h, but after heating to 55 and 72 °C for 5 days fragments of 86 bp persisted at 0.3 to 1% of the initial quantity	(Nogva and others 2000)
	Inactivation of 7.3 and 7.7 log CFU/mL <i>C. jejuni</i> in water by heating for 5 min at 55 °C, 72 °C and 100 °C	In water at 25 °C	Fragments of 86 bp persist for 60 days at approximately 100-fold decreased levels	
	Inactivation of 8.7 log CFU/mL <i>Salmonella</i> in Luria-Bertani (LB) broth by heating at 65 °C for 20 min and at 80 °C for 30 min	In LB broth at room temperature	Intact genomic DNA and 285 bp fragments persist for 18 days	
	Inactivation of 8.7 log CFU/mL <i>Salmonella</i> in LB broth by boiling for 3 min and heating at 121 °C for 15 min	In LB broth at room temperature	Intact genomic DNA is degraded after 6 days storage after boiling and within 1 day after heating at 121 °C, but DNA fragments of 285 bp persist for 10 days	(Li and others 2013)
Starvation	Starvation of 5.5 log CFU/mL <i>Aeromonas salmonicida</i>	In fresh water with sandy sediment at 13 °C	DNA was degraded to fragments < 423 bp simultaneously with the inactivation in water, but DNA fragments of 423 bp were detected for 5.5 weeks afterwards in the	(Deere and others 1996)

			sandy sediment	
		In fresh water with loamy sediment at 13 °C	DNA fragments of 423 bp were detected for 3.5 weeks after the inactivation in water and for 10.5 weeks in the loamy sediment	
	Starvation of approximately 3.5 log CFU/mL <i>Legionella pneumophila</i>	In ocean water at 16 °C	DNA fragments of 186 bp and 108 bp persist for 5 weeks after inactivation	(Palmer and others 1993)
	Starvation of 6 log CFU/mL <i>L. monocytogenes</i>	In artificial seawater at room temperature	DNA fragments of 746 bp persist after 32 days for <i>L. monocytogenes</i>	(Masters and others 1994)
	Starvation of 7 log CFU/mL <i>E. coli</i>	In distilled and tap water at 4 °C	DNA fragments of 1051 bp persist after 325 days for <i>E. coli</i>	
Irradiation	Inactivation of 6 log CFU/mL <i>E. coli</i> in phosphate buffer by UV irradiation at 254 nm at 25 °C for 1 min and 5 min	In phosphate buffer at 20 °C in the dark	DNA fragments of 147 bp persisted for 48 h	(Villarino and others 2000)
	Inactivation of 6 log CFU/mL <i>E. coli</i> by UV irradiation (12000 to 16000 µWs/cm ²) in nutrient broth	None	DNA fragments of 179 bp persist	(Josephson and others 1993)
Hydrostatic pressure	Inactivation of <i>E. coli</i> and <i>Shigella</i> in milk by high hydrostatic pressure (500 Mpa for 30 min at 25 °C)	None	Degradation of the chromosomal DNA into fragments of unspecified lengths, shown by different and weaker pulse field gel electrophoresis (PFGE) patterns	(Yang and others 2012)
Desiccation	Inactivation of 6 log CFU/mL <i>E. coli</i>	In MRD at room temperature in	DNA fragments of > 1051 bp persist	(Masters and others 1994)

	<i>coli</i> by dessication peroxide in maximum recovery diluents (MRD) by GryoVap for 20 min at room temperature	the dark	18 h after inactivation	
Acid	Inactivation of 6 log CFU/mL <i>E. coli</i> and 8 log CFU/mL <i>L. monocytogenes</i> by acid (LB broth with pH 2.0) at room temperature	None	Degradation of DNA to fragments < 1051 bp (for <i>E. coli</i>) and < 746 bp (<i>L. monocytogenes</i>) after 2 days	(Masters and others 1994)
	Inactivation of 6 log CFU/mL <i>E. coli</i> by 5 mmol/L hydrogen peroxide in MRD at 37 °C	None	Degradation of DNA to fragments of < 1051 bp in 24 to 48 h	(Masters and others 1994)
	Inactivation of 6 log CFU/mL <i>E. coli</i> by 500 mmol/L hydrogen peroxide in MRD at 37 °C	None	Degradation of DNA to fragments of < 746 bp in 48 to 960	
Disinfectants	Inactivation of approximately 9 log CFU/mL <i>Listeria monocytogenes</i> in Ringer solution with 0.5% and 2.5% Dettol	In Ringer solution at room temperature	DNA fragments of 702 bp persisted for 30 days after inactivation with 0.5% Dettol and for 90 days with 2.5% Dettol	
	Inactivation of approximately 9 log CFU/mL <i>Listeria monocytogenes</i> in Ringer solution with 5% NaOCl	In Ringer solution at room temperature	DNA fragments of 702 bp persisted for 30 days	(Herman 1997)
	Inactivation of approximately 9 log CFU/mL <i>Listeria monocytogenes</i> in	In Ringer solution at room temperature	DNA fragments of 702 bp persisted for 90 days	

	Ringer solution with 1% RBS (R. Borghgraef Solution)		
	Inactivation of approximately 9 log CFU/mL <i>Listeria monocytogenes</i> in Ringer solution with base (1% NaOH) and acid (1% HCl)	In Ringer solution at room temperature	DNA was degraded to fragments < 702 bp after 30 days
RNA			
	Inactivation of 8.7 log CFU/mL <i>Salmonella</i> in Luria-Bertani broth by heating at 65 °C for 20 min, at 80 °C for 30 min, boiling for 3 min and at 121 °C for 15 min	None	Ribosomal RNA was degraded within 1 day after and autoclaving, but intact rRNA persisted after heating at 65 °C for 20 min and even at 80 °C for 30 min mRNA fragments of 285 bp of the <i>invA</i> gene were never detected (Li and others 2013)
Heat	Inactivation of 6 log CFU/mL <i>E. coli</i> O157:H7 and <i>S. aureus</i> in brain heart infusion (BHI) broth by autoclaving (15 min at 121 °C)	In BHI broth at 37 °C	Degradation of 16S rRNA to fragments of < 323 bp immediately after autoclaving (McKillip and others 1998)
	Inactivation of 6 log CFU/mL <i>E. coli</i> O157:H7 and <i>S. aureus</i> in BHI broth by heating at 80 °C for 20 min	In BHI broth at 37 °C	Fragments of approximately 1400 bp persisted for 48 h
	Inactivation of 5 and 8 log CFU/mL <i>C. jejuni</i> cells inoculated on chicken skin	Storage of chicken skin samples at 37 °C	16S rRNA fragments of 100-170 bp persisted for 12 days with the 8 log CFU/mL (Uyttendaele and others 1997)

by heating at 100 °C for 10 min		inoculum for 24 h with the 5 log CFU/mL inoculum	
Inactivation of 7 log CFU/mL <i>E. coli</i> in LB broth by heating at 100 °C for 5 min, at 80 °C for 10 min and at 60 °C for 20 min	In LB broth at room temperature	Degradation of mRNA from the <i>rpoH</i> , <i>groEL</i> and <i>tufA</i> genes to fragments of respectively < 450 bp, < 653 bp and < 578 bp after > 2 h, but 16S rRNA fragments of 405 bp persisted for 16 h	(Sheridan and others 1998)
Inactivation of 8 log CFU/mL <i>E. coli</i> O157:H7 in skim milk by heating at 60 °C for 3 h	In skim milk at 23 °C	16S rRNA was partially degraded but fragments of 266 bp persisted after 48 h	(McKillip and others 1999)
Inactivation of 8 log CFU/mL <i>Campylobacter</i> spp. by various heat treatments ranging from 10 min at 60 °C to 90 min at 90 °C in phosphate-buffered saline (PBS)	In PBS at 37 °C	Complete degradation of mRNA after 0.5 h to 10 h	(Sung and others 2005)
Inactivation of 6 and 9 log CFU/mL <i>Listeria monocytogenes</i> in PBS by heating at 72.5 °C and 98 °C for 0, 5 and 30 min and autoclaving for 15 min	In PBS at room temperature	16S rRNA fragments of 149 bp persisted for 24 h after all treatments and mRNA fragments of the <i>inlA</i> (86 bp) and <i>rplD</i> (132 bp) genes persist after all treatments except autoclaving and heating at 98 °C for 30 min of 6 log CFU/mL cells	(Xiao and others 2012)
Inactivation of 6 log CFU/mL <i>E. coli</i> in phosphate buffer by heating	In phosphate buffer at 20 °C in the dark	16 S rRNA was completely degraded to fragments < 18 bp	(Villarino and others 2000)

	at 60 °C for 30 min and autoclaving		after 120 h	
Irradiation	Inactivation of 6 log CFU/mL <i>E. coli</i> in phosphate buffer by UV irradiation at 254 nm at 25 °C for 1 min and 5 min	In phosphate buffer at 20 °C	16S rRNA was degraded to fragments < 18 bp within 24 to 48 h	(Villarino and others 2000)
	Inactivation of 6 log CFU/mL <i>E. coli</i> O157:H7 and <i>S. aureus</i> in BHI broth by UV irradiation of 254 nm for 2.5 h at 23 °C	In BHI broth at 37 °C	16S rRNA of fragments of 1400 bp persist for 48 h	(McKillip and others 1998)
Disinfectants	Inactivation of 7 log CFU/mL <i>E. coli</i> in LB broth by exposure to 67% ethanol for 7 min	In the LB broth at room temperature	Degradation of mRNA from the <i>groEL</i> to fragments < 653 bp after 2 h and from <i>rpoH</i> genes to fragments < 450 bp after 16 h, but 16S rRNA fragments of 405 bp and <i>tufA</i> fragments of 578 bp persisted for ≥ 16 h	(Sheridan and others 1998)

Table 4: Stability of viral nucleic acids (DNA or RNA) after complete viral inactivation.

Treatment Type	Treatment conditions	Storage conditions	Nucleic acid detection	Reference
Heat	Inactivation of murine norovirus 1 (MNV-1) (> 7.8 log PFU/mL reduction) by heating at 80 °C for 2.5 min, 15 min, 30 min and 1 h	None	No intact RNA genomes were detected anymore after 2.5 min by a transfection assay RNA fragments of 154 bp were always detected at high concentrations, for example, 83% of the initial concentration of genomic copies of MNV-1/mL after 2.5 min, 59% after 15 min, 24% after 30 min and 3% after 1 h	(Baert and others, 2008)
	Inactivation of murine norovirus 1 (MNV-1) (> 3.5 log PFU/mL reduction) in water and milk by heating at 72 °C for 2 min	None	RNA fragments of 125 bp still detected (0.4 and 0.7 log genomic copies/mL reduction in water and milk respectively)	(Hewitt and others, 2009)
	Inactivation of murine norovirus 1 (MNV-1) (> 6 log PFU/mL reduction) in tissue culture medium by heating at 70 °C for 2 min	None	RNA fragments of 159 bp still detected (0.8 log genomic copies/mL reduction)	(Li and others, 2012)
	Inactivation of murine norovirus 1 (MNV-1) (> 5 log PFU/mL reduction) in tissue culture medium and 1% stool in PBS by heating at 73 °C for 3 min	None	RNA fragments of 159 bp still detected (0.8 and 0.4 log genomic copies/mL reduction in tissue culture medium and 1% stool in PBS respectively)	(Tuladhar and others, 2012)
	Inactivation of feline calicivirus (FCV) (> 4 log TCID ₅₀ /mL reduction) in	None	RNA fragments of 111 bp still detected (2.0 and 1.3 log genomic copies/mL reduction in chives and mint respectively)	(Butot and others, 2009)

	chives and mint by heating at 75 °C for 2.5 min			
	Inactivation of hepatitis A virus (HAV) in mussels (> 2.5 log TCID ₅₀ /mL reduction) by boiling for 3 min (mean internal temperature of 92 °C)	4°C for up to 1 h	RNA fragments of 206 bp still detected (2.9 log genomic copies/mL reduction)	(Hewitt and Greening 2006)
Chlorine	Inactivation of feline calicivirus (FCV) (> 3.5 log TCID ₅₀ /mL reduction) in blueberries, raspberries, strawberries and basil by 200 ppm chlorine at 18 °C for 0.5 min	None	RNA fragments of 111 bp still detected (4, > 3, 3.4, and 2.5 log genomic copies/mL reduction in blueberries, raspberries, strawberries and basil respectively)	(Butot and others, 2008)
	Inactivation of feline calicivirus (FCV) (> 5 log TCID ₅₀ /mL reduction) in tissue culture medium by 3000 and 6000 ppm chlorine at room temperature for 10 min	None	RNA fragments of 83 bp still detected (2.5 and 4.2 log genomic copies/mL reduction by 3000 and 6000 ppm chlorine respectively)	(Duizer and others, 2004)
	Inactivation of feline calicivirus (FCV) (> 3 log TCID ₅₀ /mL reduction) in 10% stool in hard water by 6000 and 7000 ppm chlorine for 15 min	None	RNA fragments of 126 bp still detected (3 log genomic copies/mL reduction)	(Poschetto and others, 2007)
	Inactivation of Poliovirus (> 3.8 log TCID ₅₀ /mL reduction) in water by 1ppm	None	RNA fragments of 197 bp and 866 bp still detected (1.5 and 2.0 log genomic copies/mL reduction, respectively)	(Shin and Sobsey, 2008)

	chlorine at 5 °C for 10 min			
	Inactivation of feline calicivirus (FCV) (> 4 log TCID ₅₀ /mL reduction) in 0.03% BSA in hard water by 48 and 66 ppm chlorine at room temperature for 30 min	None	RNA degraded to < 89 bp fragments (no longer detected by RT-qPCR)	(Nowak and others, 2011)
	Inactivation of feline calicivirus (FCV) (> 4.5 log TCID ₅₀ /mL reduction) in 10% stool on stainless steel surface by 5000 ppm chlorine for 4 min	None	RNA fragments of 229 bp still detected (1.25 log genomic copies/mL reduction)	(Park and Sobsey, 2011)
Acid	Inactivation of feline calicivirus (FCV) from 11.3 to < 1.6 log TCID ₅₀ /mL in acid marinade (pH 3.75)	Stored at 4 °C	RNA fragments of 287 bp were still detected, although at a considerably lower level (4 log decrease from 11 to 7 log genomic copies/mL); three weeks of storage at 4 °C resulted in only 1 log additional decrease of the RNA fragment detection to 6 log genomic copies/mL	(Hewitt and Greening 2004)
Oxidation	Inactivation of 10 log PFU/mL bacteriophage MS2 by singlet oxygen at a constant concentration of 4×10^{-12} M for 4 h	None	RNA fragments between 244 bp to 335 bp (12 different fragments covering the entire genome) were still detected, but at lower concentrations (reductions of 1.2 to 2.4 log)	(Pecson and others 2009)
Multiple treatments	Inactivation of feline calicivirus (FCV) from 9.4 to < 1.6 log TCID ₅₀ /mL within 1 week of storage at 4 °C in mussels after a commercial marination	Stored at 4 °C	RNA fragments of 287 bp were still detected, although at a considerably lower level (3 log decrease from 8 to 5 log genomic copies/mL); three weeks of storage at 4 °C resulted in only 1 log additional decrease of the RNA fragment detection to 4	(Hewitt and Greening 2004)

	process combining heat and acid treatment, for example, boiling in water for 37 s, steaming for 188 s, cooled immediately on ice, shucked, immersed in acid marinade (an acetic acid solution with pH 3.75)		log genomic copies/mL	
Incubation in seawater	Poliovirus (4.6 log PFU/mL) was completely inactivated in natural seawater	22 and 30 °C for 30 days	RNA fragments of 197 bp were no longer detected in natural seawater after 10 days at 22 °C and 3 days at 30 °C, despite the presence of 1 to 2 log PFU/mL	(Wetz and others 2004)
	Poliovirus (4.6 log PFU/mL) was completely inactivated in filter-sterilized seawater	22 and 30 °C for 30 days	RNA fragments of 197 bp were detected after ≥ 60 days at 22 °C and 37 days at 30 °C, so RNA persisted at least 1 week after the complete inactivation of polioviruses in filter-sterilized seawater	(Wetz and others 2004)
Incubation in liquid dairy manure	Murine norovirus 1 (MNV-1) was completely inactivated (> 2 log PFU/mL reduction) in liquid dairy manure	4°C for 20 days	RNA degraded to < 318 bp fragments (no longer detected by RT-qPCR)	(Wei and others, 2010)

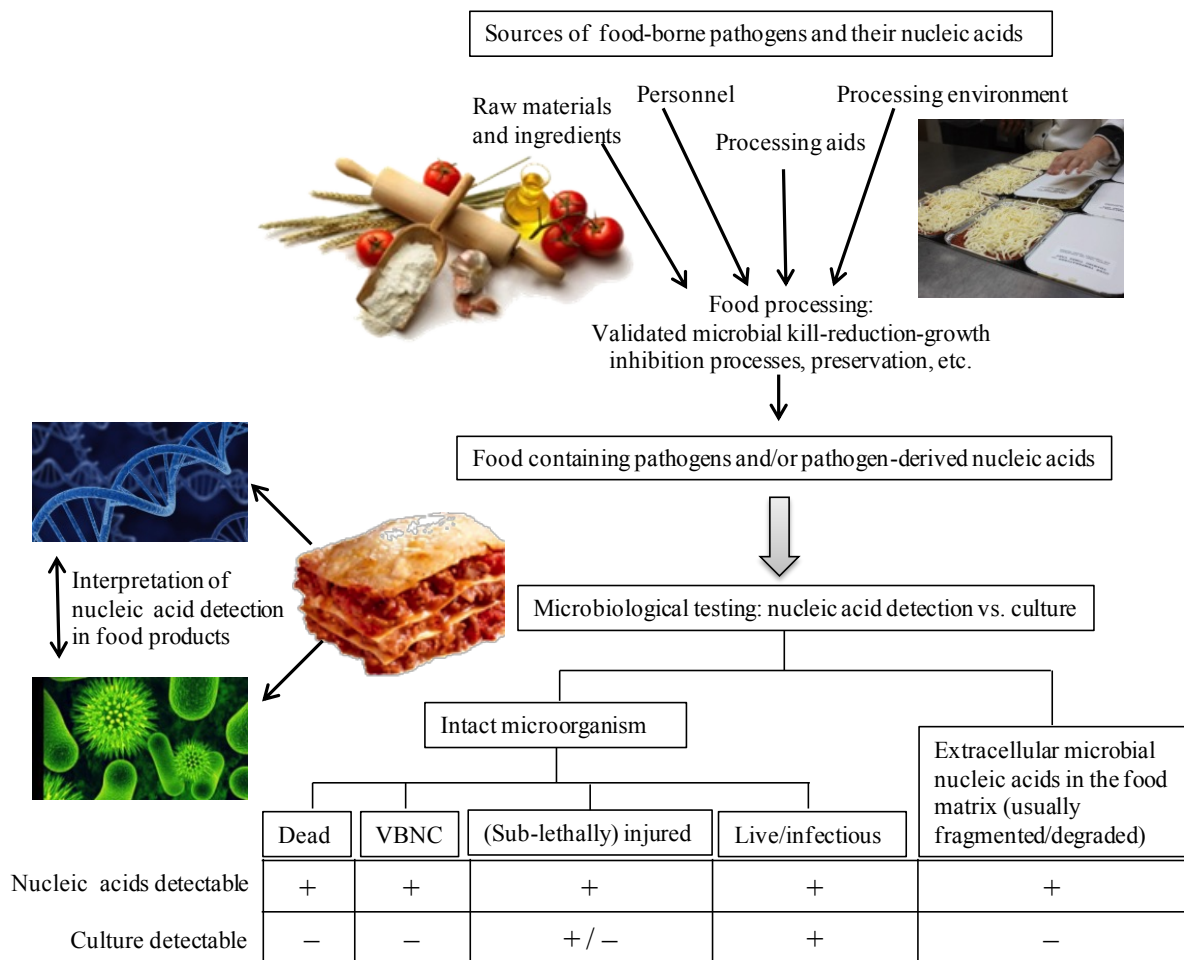


Figure 1: Overview of possible contamination routes of food with microbial pathogens and/or their nucleic acids and the possible consequences for detection with nucleic acid-based and culture-based methods.

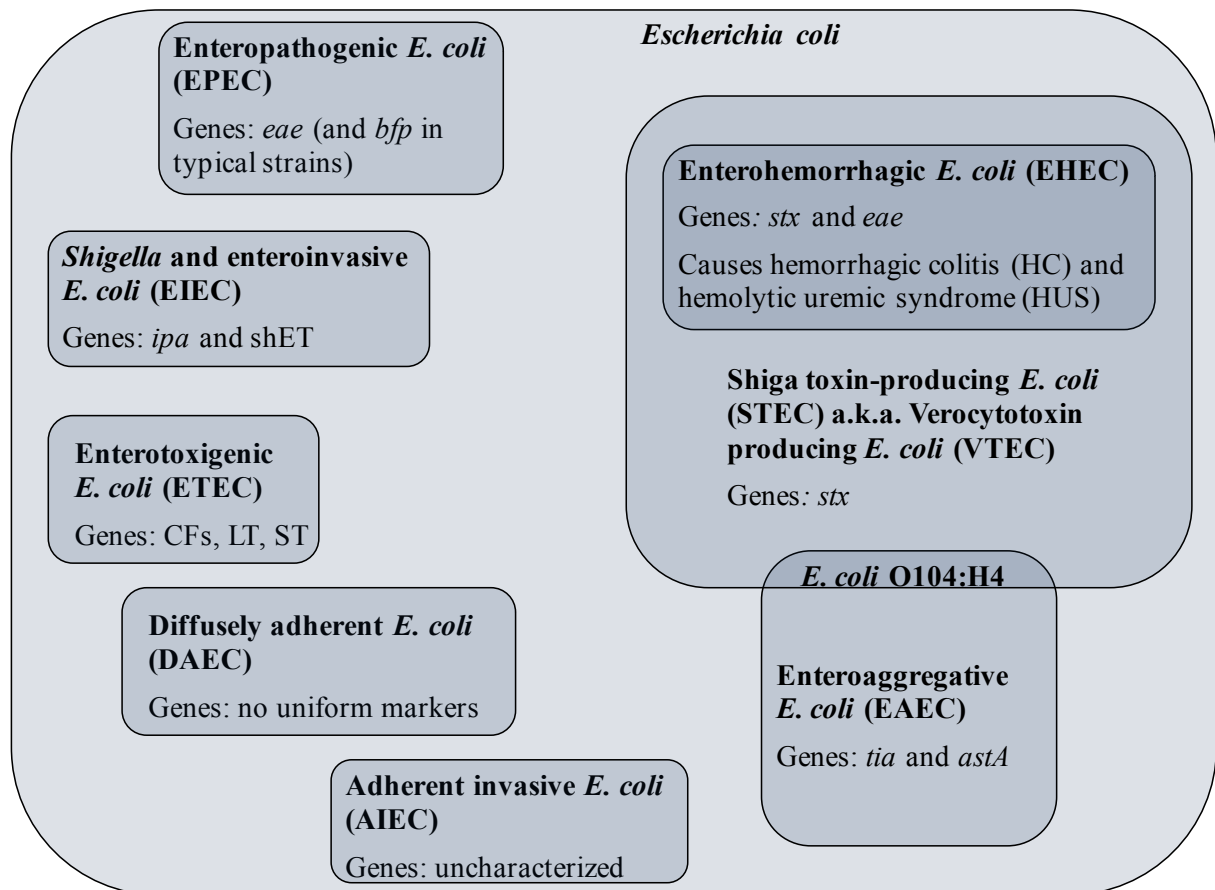


Figure 2: Different pathotypes of *E. coli* and their characterizing genetic determinants; *stx*: verocytotoxin/Shiga toxin, *eae*: intimin, *bfp*: bundle-forming pili, *ipa*: invasion plasmid antigens, *tia*: toxigenic invasion loci A, *astA*: plasmid with enteroaggregative heat-stable toxin, shET: *Shigella* enterotoxin, CFA: colonization factor antigens, ST: heat-stable enterotoxin, LT: heat-labile enterotoxin.

<p>Culture and PCR negative</p> <ul style="list-style-type: none"> • No nucleic acids • No microorganisms 	<p>Culture positive and PCR negative</p> <ul style="list-style-type: none"> • Recalcitrant microorganisms (spores, biofilms, internalization) <p>→ Possible solutions</p> <ul style="list-style-type: none"> • Sample preparation optimization
<p>Culture negative and PCR positive</p> <ul style="list-style-type: none"> • Free nucleic acids • Non-infectious viruses • Dead bacteria • Sub-lethally injured and VBNC bacteria <p>→ Possible solutions</p> <ul style="list-style-type: none"> • Culture confirmation • Prior EMA/PMA treatment • Secondary and/or large region amplification • Prior enzymatic treatment with nucleases • Prior binding assays • Prior enrichment/resuscitation • (m)RNA detection 	<p>Culture and PCR positive</p> <ul style="list-style-type: none"> • Infectious viruses • Viable bacteria

Figure 3: Interpretation of nucleic acid detection results and possible solutions to cope with interpretation problems

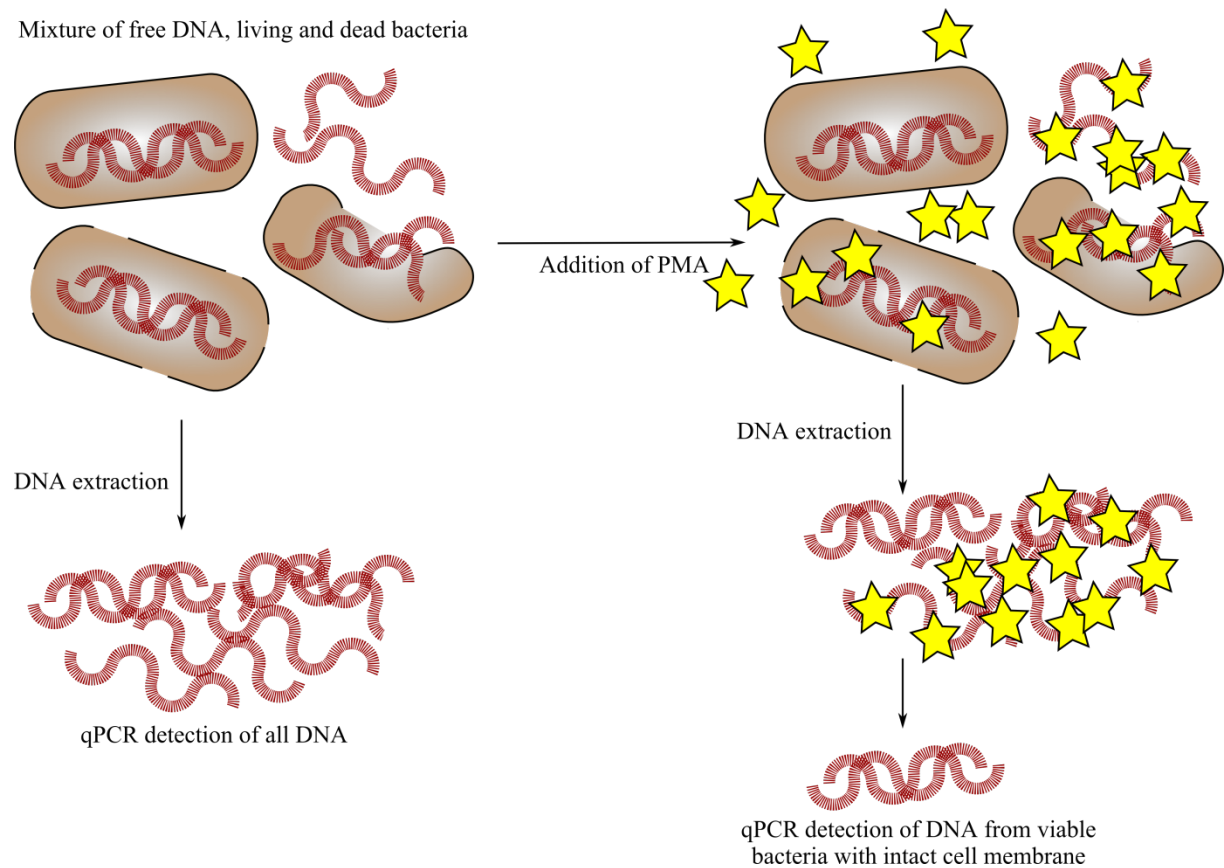


Figure 4: Principle of EMA/PMA-PCR to specifically detect nucleic acids from viable bacteria, based on the exclusion of PMA from viable bacteria due to their intact cell membrane.

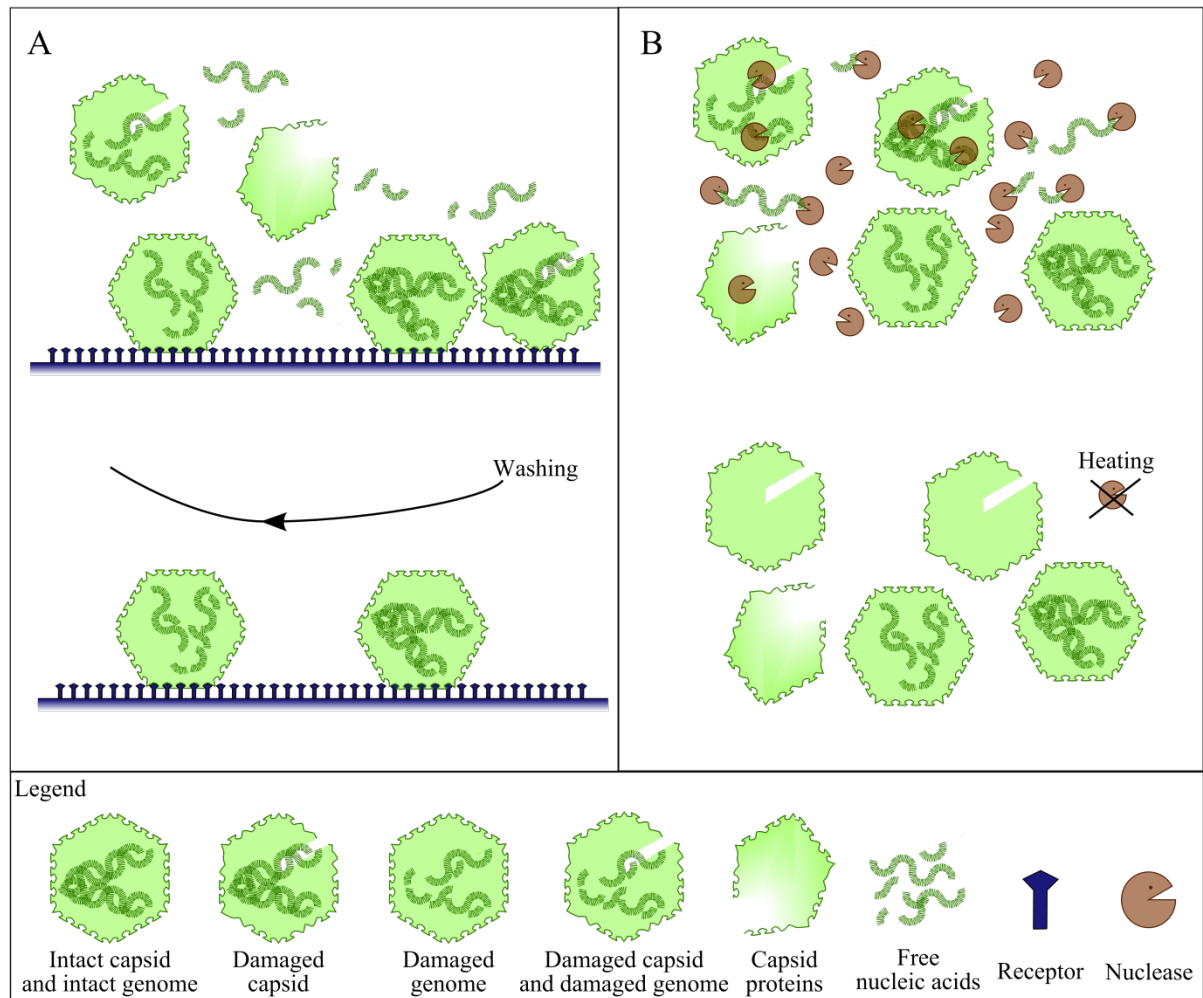


Figure 5: Alternative virus detection strategies to target infectious virus particles based on capsid integrity. (A) Conducting a binding assay prior to (RT)-(q)PCR detection aims to retain virus particles with intact capsids for subsequent nucleic acid extraction while unbound defective particles and free nucleic acids are removed by washing (B) Enzymatic pre-treatment nucleases attempts to degrade all accessible nucleic acids, namely, free nucleic acids and nucleic acids from viruses with damaged capsids.

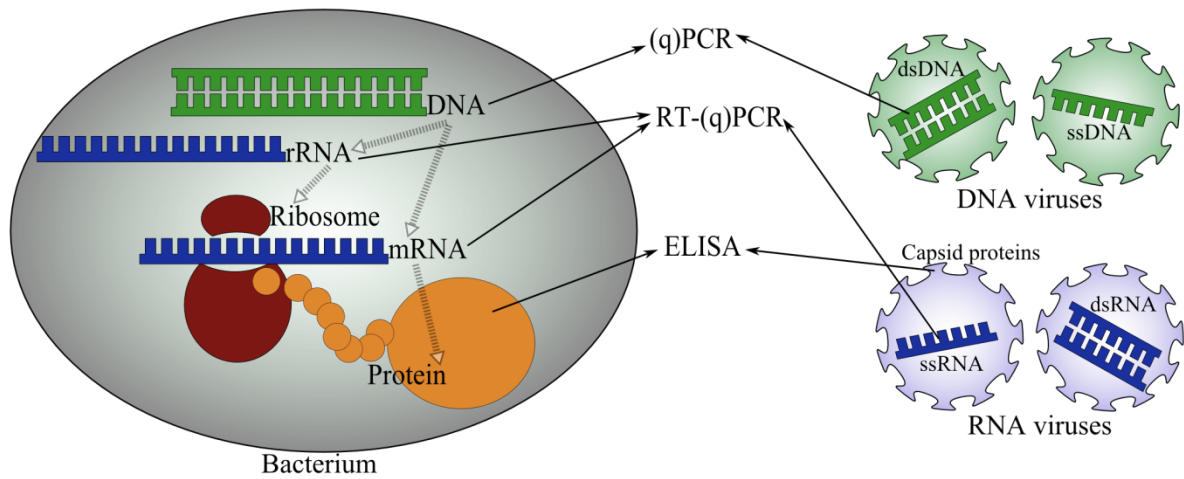


Figure BOX 1: Detection of DNA by (q)PCR, of viral RNA and bacterial mRNA and rRNA by RT-(q)PCR and proteins by ELISA.

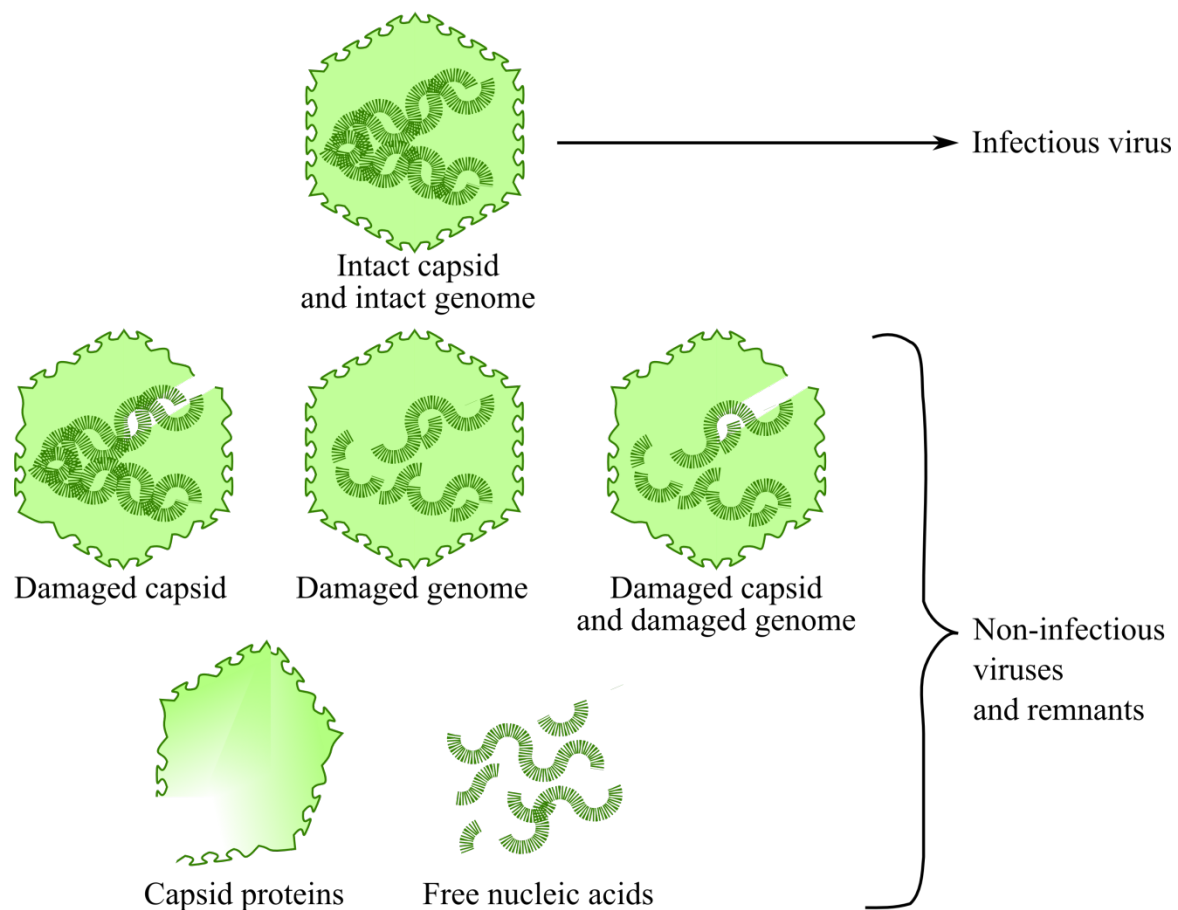


Figure BOX 3: Different virus particles and remnants present in virus suspensions.